Institute of Paper Science and Technology
Atlanta, Georgia

ANNUAL PROGRAM REVIEW

FOREST BIOLOGY

Slide Book

March 25-26, 1999

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FOREST BIOLOGY ANNUAL RESEARCH REVIEW AGENDA

Thursday, March 25, 1999
Forest Biology Annual Program Review (Room 114)

7:45 A.M.	Coffee and Donuts	
8:00	Welcome, introduction, antitrust statement	McCullough
	IPST Update	Baum

F-010 (SOFTWOODS) DUES FUNDED CONSORTIUM PROJECTS
(1.2 Senior, 3.5 Support Staff)

08:30	F-010 Mass Clonal Propagation of Improved Conifers	Pullman
	Summary of Accomplishments Since Last Meeting	
	Goals	
	Personnel	
	Grants	
	Research Findings	
09:00	Softwood Somatic Embryogenesis	
	Initiation	Pullman
	Dissecting the variation in initiation	MacKay
	Culture Survival	Peter
	Improved Embryo Yield & Quality	
	Improvements Based on Metals Analysis	Pullman
	Plating Process Optimization Per Genotype (ABA)	Vales / Peter
10:30	Break	
10:45	Conversion Update	Pullman
	Progress in Protocol Development & Targets	Pullman
11:00	Zygotic Embryogenesis	
	Free Amino Acids Update	Zhang
	Zygotic Germination Update	Peter
12:00	Lunch	

1:00	Molecular Biology - Softwoods Gene Expression in Pine Embryos Understanding the Role of Suspensor Tissue in Embryo Development	MacKay
	Somatic Embryos - Stage-specific Markers	Cairney, Johns

EXTERNALLY FUNDED & STUDENT PROJECTS RELATED TO F010

1:40	Gene Expression During Embryogenesis Rapid Gene Expression Comparisons	Ge
	Using Gene Comparisons to Improve Somatic Embryo Quality	Cairney
2:20	Somatic Embryo - Early stage-specific markers	Ciavatta
3:00	Break	
3:15	Future goals of softwood research, Program evaluation	Pullman, Peter, Cairney, MacKay

F-011 DUES FUNDED CONSORTIUM PROJECTS (0.33 Senior, 0.5 Support)

3:45	F-011 Fundamental Biological Mechanisms: Improved Stem Growth Rates and Fiber Properties	
	Summary of Accomplishments Since Last Meeting, Goals, Personnel, Grants	Peter
	Future Goals - Building a Program Towards Fiber Modification, Program Evaluation	Peter

EXTERNALLY FUNDED & STUDENT PROJECTS RELATED TO F011

4:15	Building a Transformation System for Loblolly Pine	Peter
	Microfibril Angle Update	Peter / Benton
	AQ Update	Peter / Meng
	CAD-Defficient Trees for Improved Pulping	MacKay
	New DFRC Project – Genetic Manipulation of Lignin Future Goals	MacKay

5:15	Poster Session (Start, Continues after Dinner)	Visiting Co-PIs
6:00	Dinner / Poster Session	Visiting Co-PIs Postdocs Students

Friday, March 26, 1999
Forest Biology PAC Meeting (Room 114)

7:45 A.M.	Coffee and Donuts	
8:00	Grant Proposal Activity, Student Research, Publications	Pullman, Cairney Peter, MacKay
8:45	Comments on Research Programs, Questions, Discussion, Issues	PAC
10:00	Break	
10:15	Continued Discussion	
11:00	Presentation at RAC Forest Biology Review (11/17/98)	McCullough
11:15	RAC Developments,	Malcolm (for Matthews)
11:30	Third Party Agreements for Use of IPST Technology Role of Non-IPST Patents in IPST Research Update on Project ROCIT AF&PA Agenda 2020 Sustainable Forestry Proposals	Malcolm
12:00	Adjourn (Lunch will be available at 12:00)	

IPST DUES FUNDED RESEARCH CONSORTIUM
1998-1999

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Status Report for Project F010

Gerald Pullman
John Cairney
Gary Peter
John MacKay
Lin Ge
Barbara Johns
Shannon Johnson
Paul Montello
Christina Perfetti
Teresa Vales
Yalin Zhang

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Annual Program Review - Forest Biology March 25-26, 1999

Project Title: MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Project Code: SFTWD

Project Number: F-010

Research Line/RoadMap: Improve the fiber productivity of North American lands so that they are competitive in the world pulpwood market. Develop improved trees via genetic manipulation.

Project Objective: Develop reliable cell & tissue culture systems for the mass clonal propagation of genetically improved softwoods.

Project Budget 97/98: $440,000 (1.1 professional, 4 support staff)
Allocated as Matching Funds: 11%)

Project Staff: Faculty/Associate Scientist: Gerald Pullman, John Cairney, Gary Peter, John MacKay; Postdoc: Lin Ge, Staff: Barbara Johns, Shannon Johnson, Paul Montello (open), Xiarong Feng (open), Theresa Vales, Yalin Zhang, Christina Perfetti, temporary staff

Forest Biology Annual Program Review March 25-26, 1999 -Funded Work & Student Research Related to F-010

EXTERNAL RESEARCH FUNDING

TIP^3 (The Georgia Consortium for Technological Competitiveness in Pulp and Paper) "Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies". **$144,377**
TIP^3 (The Georgia Consortium for Technological Competitiveness in Pulp and Paper) "Genetic Transformation Methods for Southern Pine". **Awarded $55,000**.

STUDENT RESEARCH

Tyler Miller (M. Sc.) First Year Student. Title: Gene Identification in Loblolly Pine.

Douglas Mancosky (M.Sc.) Second Year Student. Title: Temporal and Spatial Analysis of Gene Expression During Pine Zygotic Embryogenesis.

Vincent Ciavatta (Ph. D.) Title: Analysis of Gene Expression During Development of Somatic and Zygotic Embryos.

Stephen Van Winkle (Ph. D.) Title: An investigation into an unsuccessful tissue culture medium: Determining the role of activated charcoal.

Annual Program Review - Forest Biology March 25-26, 1999 -Summary of Results
(Brackets show support from student research or outside projects.)

Initiation, the first step in the embryogenesis process, been substantially improved. Initiation rates on a new medium (889) averaged 17.9% across 32 ½-sib families. This is approximately double the average initiation rate for the past two years.

Analysis of genetic and environmental sources of variation in initiation is helping to understand the driving forces that may control initiation.

Studies into the survival of initiated cultures after direct transfer into liquid multiplication media show:

- Overall success of direct transfer into multiplication media 16 in 1998 was ~30%.
- Within ½ sib families we have found a good correlation with the starting mass of the initiated genotype and its successful transfer and growth in multiplication media. 80-90% of initiated genotypes survived if there starting mass was >0.2g.
- Strong ½ sib family/genotype effects have been observed for survival of the initiated somatic embryos.

Ph.D. student research has shown that activated carbon significantly alters the final pH of tissue culture medium. Changes in pH in turn alter the availability of specific ions and hormones resulting in unwanted excess or deficiency. (Student research)

Annual Program Review - Forest Biology March 25-26, 1999 -Summary of Results

Optimizing the number of early stage somatic embryos plated and the ABA levels leads to dramatic increases in yield of cotyledonary embryos.

- We have observed that fewer embryos develop on plates when one ml of settled volume is plated than on plates with 0.25 – 0.5 ml of settled volume. This suggests that in our typical procedure too many early stage embryos per plate limit maturation efficiencies.
- When the density and ABA levels are optimized relative to each other the efficiency of early stage embryos that mature into cotyledonary embryos is > 75% with our system for multiple genotypes.

Comparisons of germinating somatic and natural seed embryos confirm that our somatic embryo functions normally but is immature. Current somatic embryos behave similar to zygotic embryos that have proceeded through approximately ½ of their development cycle.

Tissue culture media formulation by a combination of metal analysis during natural embryo development and the comparison of metals found in natural and somatic embryos at different times is yielding beneficial results.

- Metal analyses of seed indicate that metal targets are similar regardless of tree location or genetics.
- Analyses of embryo & surrounding tissues across developmental show sequence patterns.
- Analyses of somatic embryos show excess or deficiencies in ion content.
- Media changes based on the above elemental analyses produce statistically significant improvements in embryo yield.

Annual Program Review - Forest Biology March 25-26, 1999 -Summary of Results
(Brackets show support from student research or outside projects.)

Ph.D. student research has isolated cDNAs expressed at early stages of development. These can act as markers to follow early embryo development. Note at this point anatomical features may be small and difficult to see. (Student research)

Molecular biology gene expression techniques are working well in the laboratory. IPST is becoming a world leader in adapting and applying this technology to plant embryogenesis.

- Image analysis equipment has been purchase to permit accurate quantification of changes in mRNA level over the course of embryo development
- Quantification procedures have been developed which will ensure accurate measurement of these data
- Work has commenced on establishing a database of mRNA levels for around 450 genes over the course of embryo development (4130)
- Methods for isolating 'full-length' cDNA clones, rapidly, without the need of cloning or the use of radioactivity have been developed. (student research)
- Several cDNAs for gene expressed early in embryogenesis have been identified and cloned – these can serve as markers for early development (student research and F010)
- Methods for Northern Analysis using very small amounts of total RNA (0.5microgram) are being developed

Annual Program Review - Forest Biology March 25-26, 1999 -Summary of Results

(Brackets show support from student research or outside projects.)

The role and development of the suspensor in loblolly pine embryos is being investigated through gene expression experiments. The suspensor is thought to play an important role in embryo nutrition and regulation of embryo growth and development. Information indicates the importance of suspensor-formed storage proteins, likely involved in nutrition during early embryo development.

- Over 750 cDNAs representing genes active in the suspensor have been cloned and analyzed.
- Several of these cDNAs were confirmed to be specific or more abundant in the suspensor of early stage embryos
- Sequencing cDNAs has identified genes for several seed storage proteins and embryogenesis proteins that are abundant later in development.
- Screening of cDNAs has also been extended to the megagametophyte.

Faculty, staff, and students in the Forest Biology with position and project orientation.

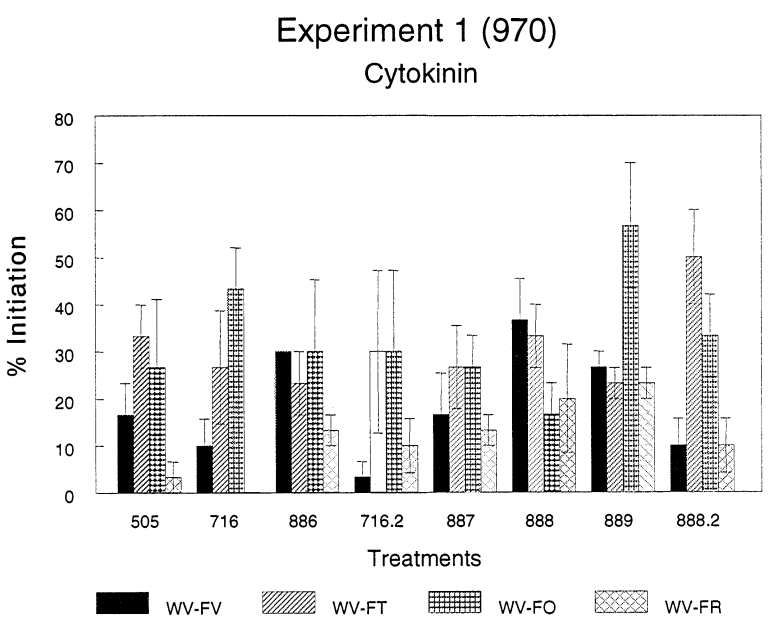
Person	Position	Orientation
Faculty&Senior Staff		
John Cairney	Assistant Professor	Molecular Biologist / Molecular Biology of Trees
John MacKay	Associate Scientist	Geneticist/Biochemist/Molecular Biology / SE, HW,
Gary Peter	Assistant Professor	Plant Physiologist, Molecular Biologist / SE, HW,
Gerald Pullman	Professor	Plant Pathologist, Physiologist / Clonal Propagation of Conifers
Postdoctoral Fellows		
L. Destefano (left 3/1599 open)	Postdoctoral Fellow	Transformation in L Pine (GA Consortium Project)
Lin Ge	Postdoctoral Fellow	Gene Expression in Embryos (GA Consort Project)
Huabin Meng	Postdoctoral Fellow	AQ Biochemistry & Gene Transfer (Ag 2020 DOE)
open	Postdoctoral Fellow	F011
Technical Staff		
Teresa Vales	Senior Technician	Clonal Propagation of Conifers
Paul Montello, left 2/28/98, open	Senior Technician	Clonal Propagation of Conifers
Yalin Zhang	Technician III	Clonal Propagation of Conifers
Xiagrong Feng, left 6/30/98, open	Technician III	Clonal Propagation of Conifers
Barbara Johns	Senior Technician	Molecular Biology of Forest Trees
Christina Perfetti	Technician III	Molecular Biology of Forest Trees
Shawn Coy	temporary staff, pt	Clonal Propagation of Conifers
Jessica Halpin	temporary staff, pt	Clonal Propagation of Conifers
Students		
Vincent Ciavatta	Ph.D. Candidate	Use of molecular biology to improve somatic embryos
Mike Sullivan	Ph.D. Candidate	Molecular approaches to isolate and characterize b-1,4-xylan synthase(s)
Steve Van Winkle	Ph.D. Candidate	Effects of Activated Charcoal on Tissue Culture Medium
Doug Benton	Masters Candidate	Microfibril angle measurement using differential interference contrast microscopy
Kristina Knutson	Masters Candidate	Fungal production of anthraquinones.
Tyler Miller	Masters Candidate	Embryogenic gene isolation
Matt Roberts	Masters Candidate	The Effect of Silvicultural Treatment on Variation in Microfibril Angle in Southern Pines
Douglas Mancosky	Masters Candidate	Temporal and Spatial Analysis of Gene Expression During Pine Zygotic Embryogenesis

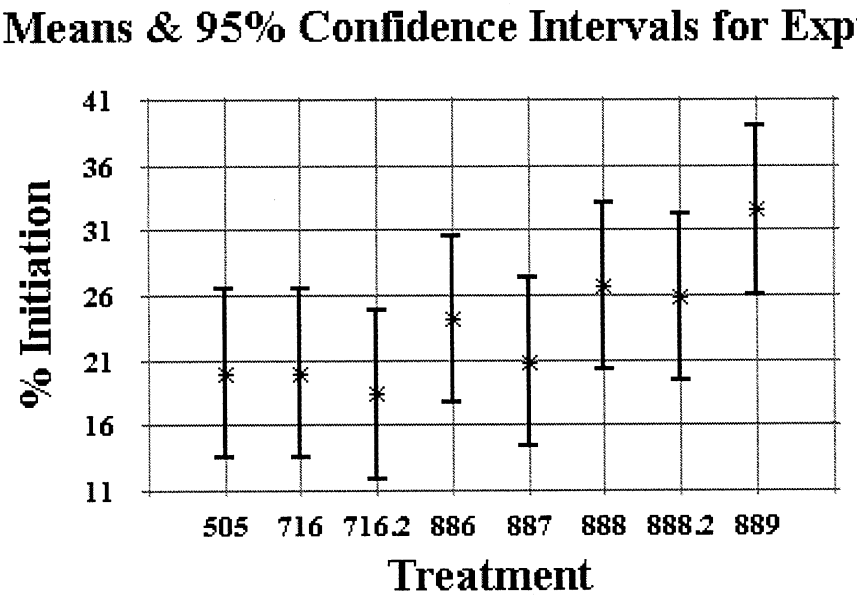
Somatic Embryogenesis Initiation

Gerald Pullman
Yalin Zhang
Nazima Allaudeen
Anneil Basnandan
Sean Coy
Chris Culbreth
Jessica Halprin
Shannon Johnson
Allison Snow

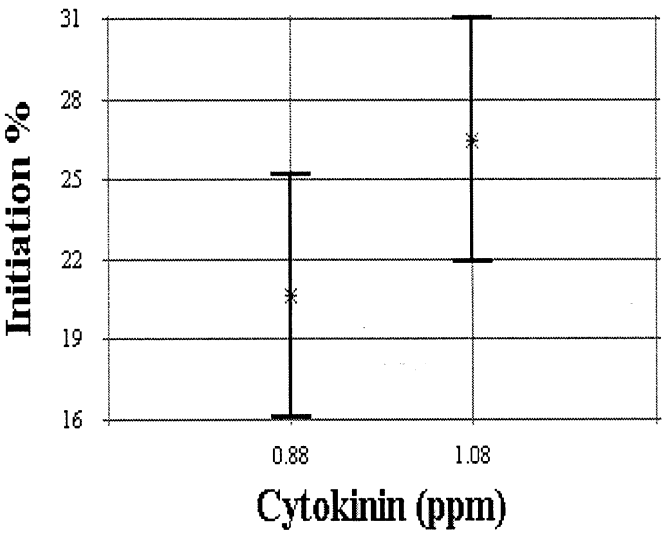
Experiment 970

Media	ABA	AgNO3	Cytokinin	cGMP	Tape
505	0 ppm	0 mM	0.88 ppm	0 μM	parafilm
716	1	20	0.88	0	parafilm
886	1	20	0.88	10	parafilm
716a	1	20	0.88	0	3M Tape
887	0	0	1.08	0	parafilm
888	1	20	1.08	0	parafilm
889	1	20	1.08	10	parafilm
888a	1	20	1.08	0	3M Tape



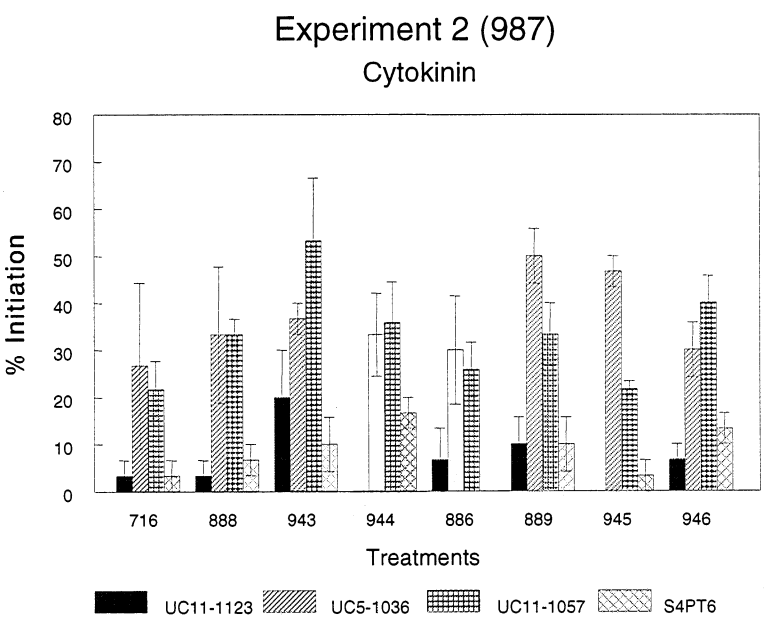


Experiment 970 Final Means & Confidence Intervals



Experiment 987

Media	Cytokinin (ppm)	ABA (ppm)	AgNO3 (mM)	cGMP (μM)
716	0.88	1.0	20	0
888	1.08	1.0	20	0
943	1.24	1.0	20	0
944	1.50	1.0	20	0
886	0.88	1.0	20	10
889	1.08	1.0	20	10
945	1.24	1.0	20	10
946	1.50	1.0	20	10



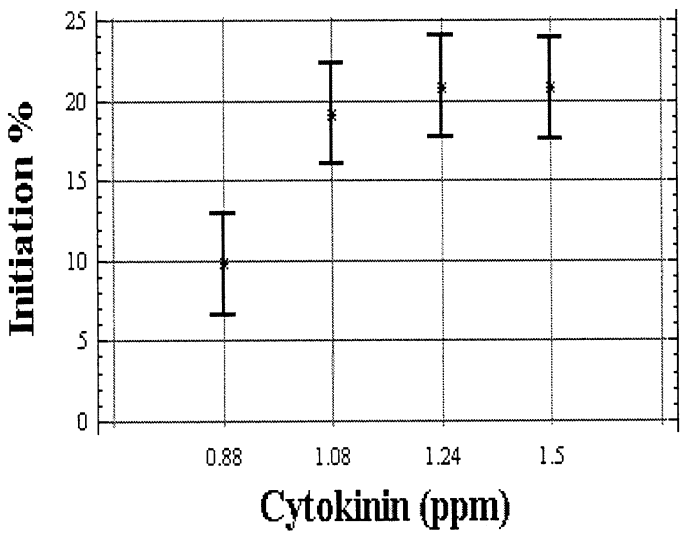


Table 1. Loblolly pine cone collection mother trees with 1995, 1996, and 1997 and 1998 initiation rates on medium 505 and improved medium 889.

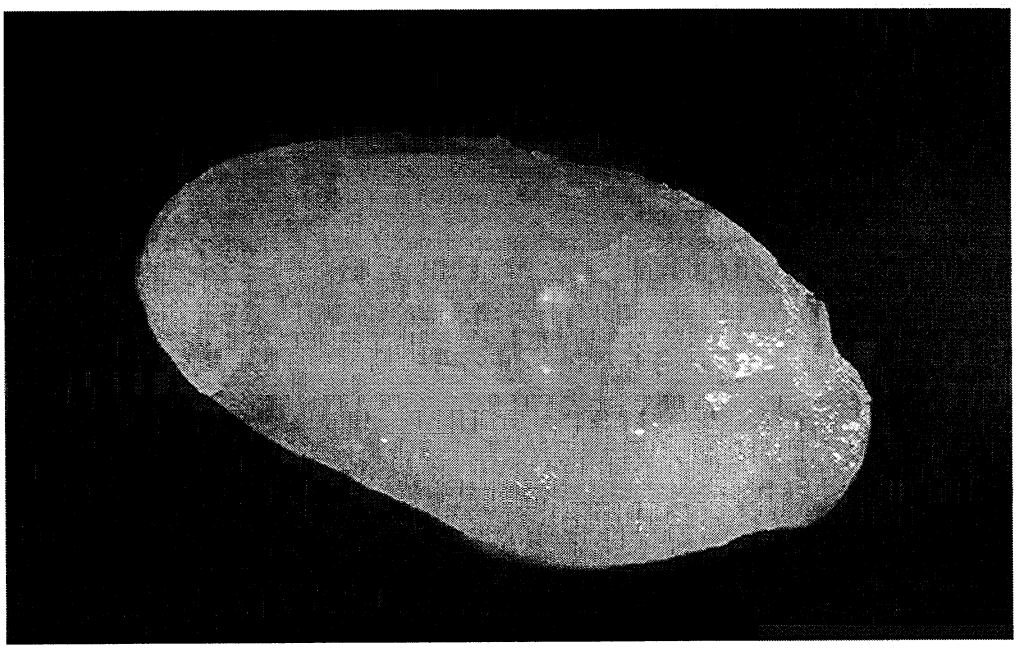
Tree Identification	Initiation % 1995	Initiation % 1996	Initiation % 1997	Initiation % 1998 Medium 889			1998 Trial Averages
	Medium 505	**Medium 505**	Medium 505	Trial 1	Trial 2	Trial 3	
BC-1 (S4PT6)		6.7%		10			10
BC-2	10%	0.0%					
BC-3		3.2%					
BC-5		9.3%					
BC-8							
BC-9	17%	10.7%					
C7-2			0				
C7-88			1.7				
C7-100			0				
C8-76			4.8				
C10-14			0				
C10-38			6.7				
UC5-1036	32%	7.9%	3.3	50	16.7	10	25.6
UC5-1507			8.9				
UC7-1037	10%						
UC7-1051		4.5%	3.3	6.7	0		3.4
UC10-1027	33%	13.8%					
UC10-5	3.3%						
UC10-33	12%						
UC11-1055		3.3%	2.2	15.6	16.7		16.2
UC11-1057		15.3%	23.7	33.3	10	20.8	21.4
UC11-1066		10.0%					
UC11-1069		4.4%					
UC11-1123			1.3	10	0	0	3.3
UC18-120				0			0
UC18-1212			23.6	26.7	20	16.7	21.1

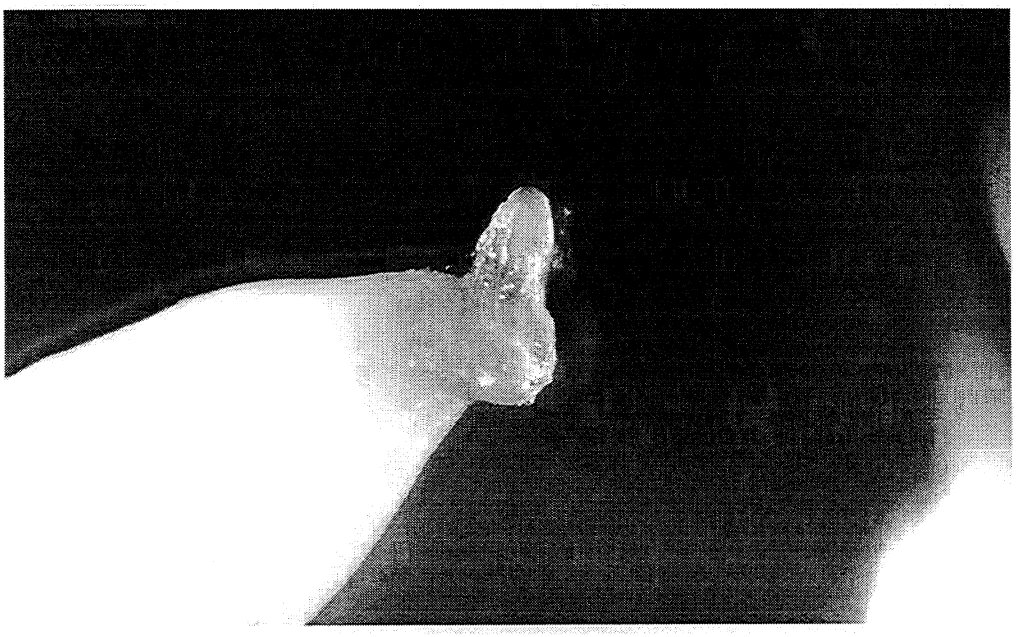
TTC 1-11				0			0
TTC 3-1				11.7			11.7
TTC 3-17				6.7			6.7
TTC 7-3				0			0
TTC 7-100				1.7			1.7
TTC 18-102				1.9			1.9
WV A4				0			0
WV B4				6.6			6.6
WV C4				18.8			18.8
WV D4				8.9			8.9
WV E4				0			0
WV F	F2 11%			F4 11.1			11
WV FO				56.7*	50*		53.4
WV FR				23.3*			23.3
WV FT				23.3*	33.3 *		28.3
WV FU				16.7*			16.7
WV FV				26.7*			26.7
WV G	G2, stages late			G4 0			0
WV H-2, 3, 4		6.7%	26.7	H4 31.1	20	36.7	29.3
WV I-2, 3	15%	3.3%	12.2				
WV J-2, 3		7.0%	10.3				
WV K-2, 3		19.0%	8.9				
WV L-4				26.7	26.7	23.3	25.6
WV M4				36.7			36.7
WV N4				56.7	26.7		41.7
WV O4				46.7	10		28.4
M 9-1019				14.4	0		7.2
Overall	16%	7.4%	8.5%	17.9			15.2

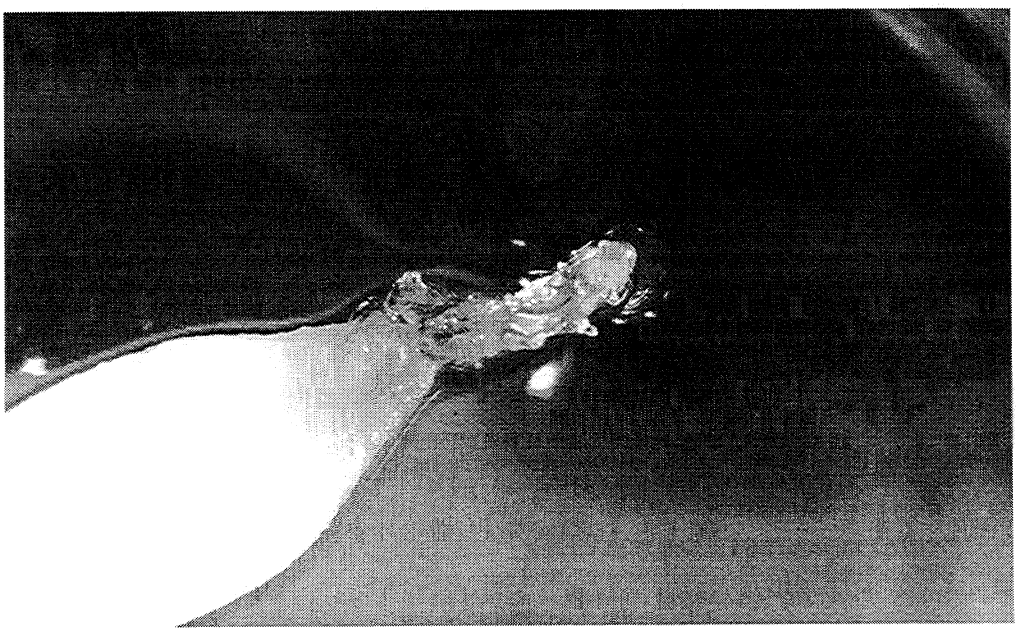
BC = Boisie Cascade, C = Champion, UC = Union Camp, WV = Westvaco, M = Mead, * Winter Initiation with Cones from Brazil.

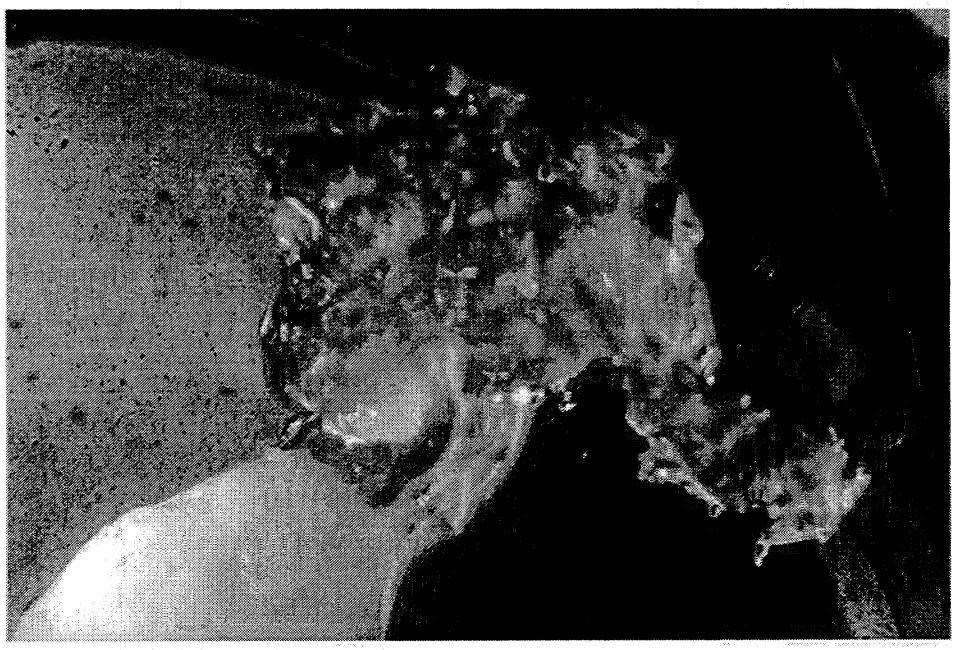
Table 2. Initiation progress over the past six years with comparisons of initiation rates on best media and across all summer initiations.

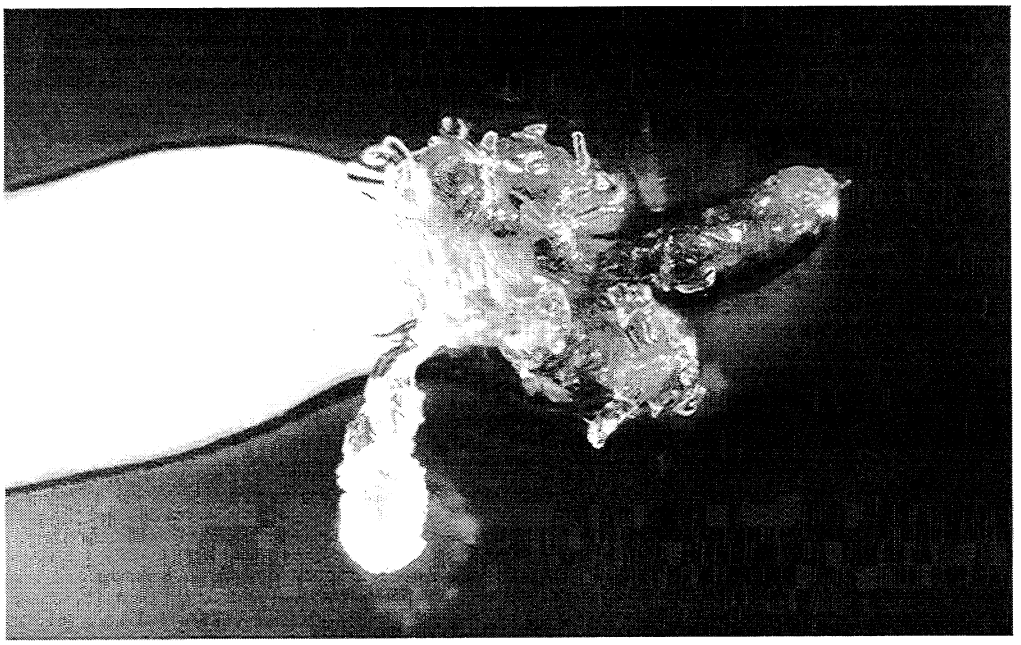
Year	Initiation in Best Medium		Initation Rates Across All Summer Expts.	
	Medium	Initiation %	Initiations / Explants	Overall Initiation %
1993		< 1%		< 1%
1994		< 1%		< 1%
1995	505	16.0%	436 / 6400	6.8%
1996	505	7.4%	765 / 13440	5.7%
1997	505	8.5%	752 / 9890	7.6%
1998	889	17.9%	1437 / 9870	14.6%

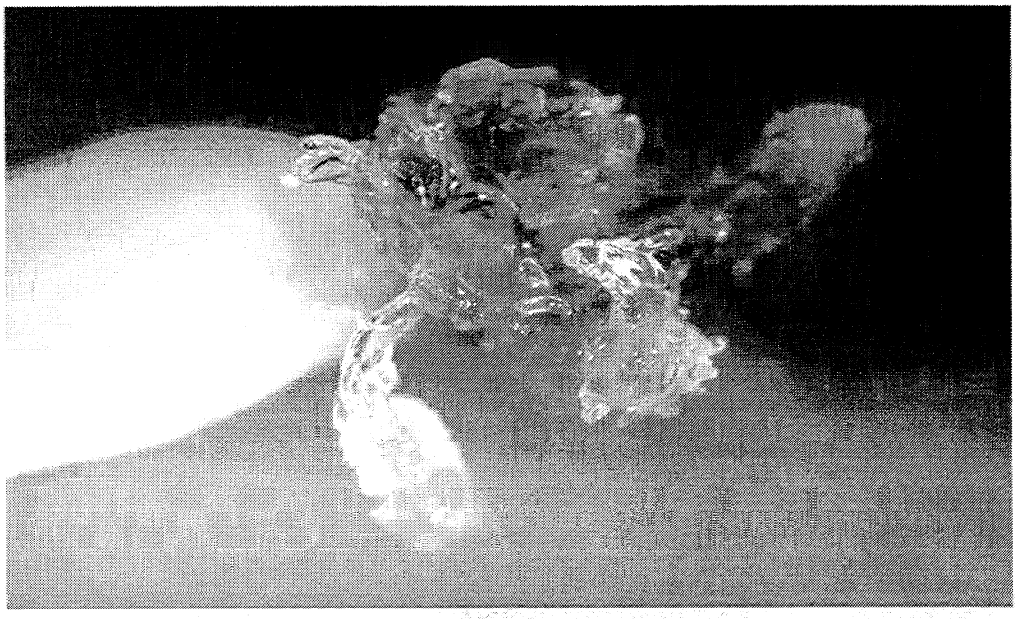


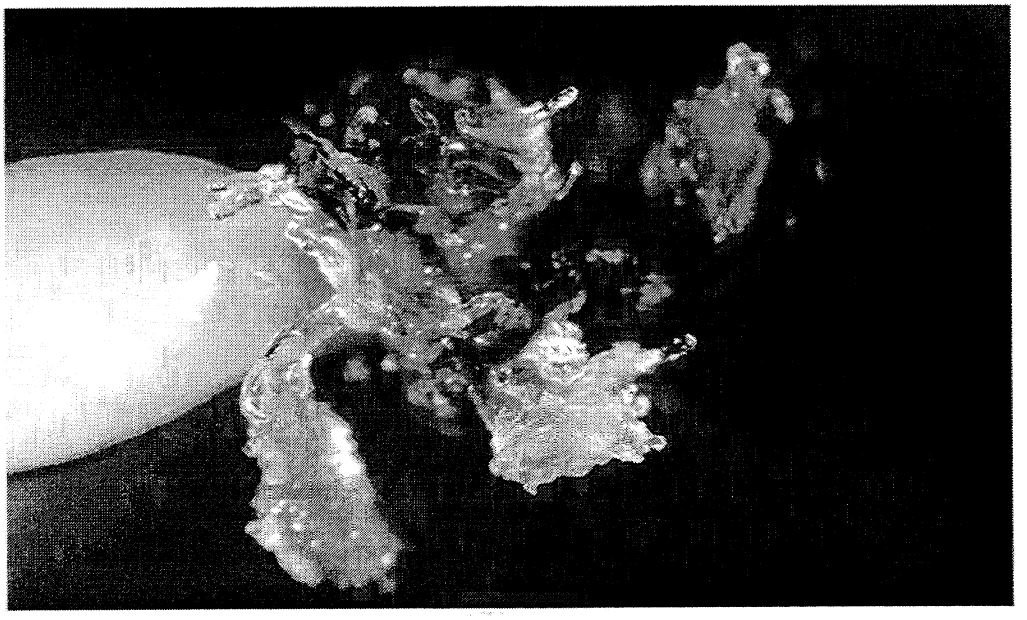


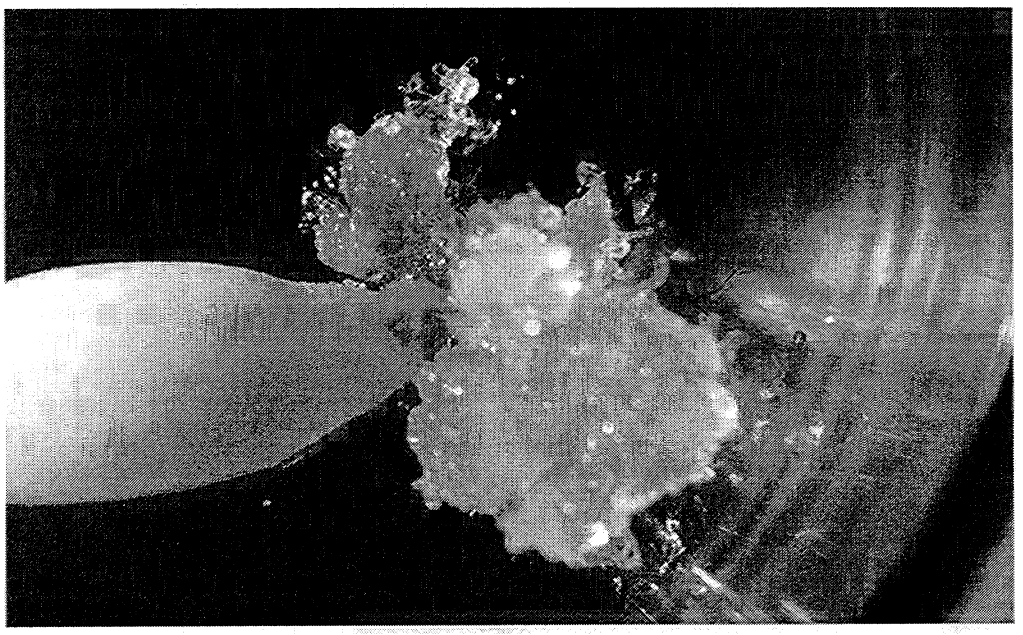












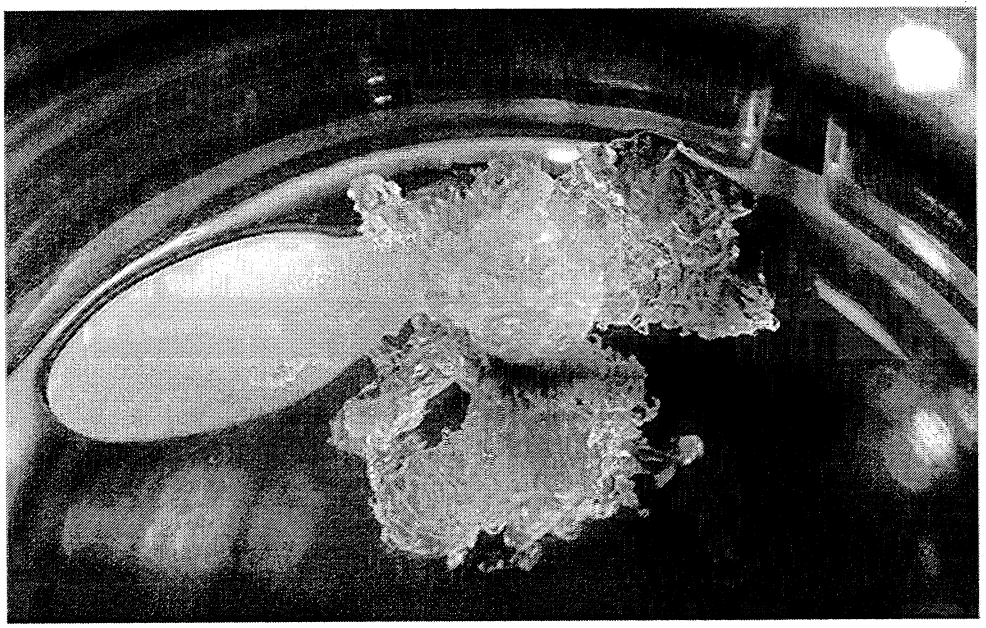


Table 2. Media composition for initiation medium 505, 716, and 889.

Components	505	716	889
NH_4NO_3	200.0	200.0	200.0
KNO_3	909.9	909.9	909.9
KH_2PO_4	136.1	136.1	136.1
$Ca(NO_3)_2 \cdot 4H_2O$	236.2	236.2	236.2
$MgSO_4 \cdot 7H_2O$	246.5	246.5	246.5
$Mg(NO_3)_2 \cdot 6H_2O$	256.5	256.5	256.5
$MgCl_2 \cdot 6H_2O$	101.7	101.7	101.7
KI	4.15	4.15	4.15
H_3BO_3	15.5	15.5	15.5
$MnSO_4 \cdot H_2O$	10.5	10.5	10.5
$ZnSO_4 \cdot 7H_2O$	14.668	14.668	14.668
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.125	0.125
$CuSO_4 \cdot 5H_2O$	0.1725	0.1725	0.1725
$CoCl_2 \cdot 6H_2O$	0.125	0.125	0.125
$AgNO_3$	--	3.398	3.398
$FeSO_4 \cdot 7H_2O$	13.9	13.9	13.9
Na_2EDTA	18.65	18.65	18.65
Maltose	15,000	15,000	15,000
myo-Inositol	20,000	20,000	20,000
Casamino Acids	500	500	500
L-Glutamine	450	450	450
Thiamine•HCL	1.0	1.0	1.0
Pyridoxine•HCL	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Glycine	2.0	2.0	2.0
NAA	2.0	2.0	2.0
BAP	0.45	0.45	0.55
Kinetin	0.43	0.43	0.53
Activated Charcoal	50	50	50
ABA*	--	1.0	1.0
GMP*	--	--	10uM
Gelrite	2,000	2,000	2,000
pH	5.7	5.7	5.7

* ∞ is added filter sterilized.

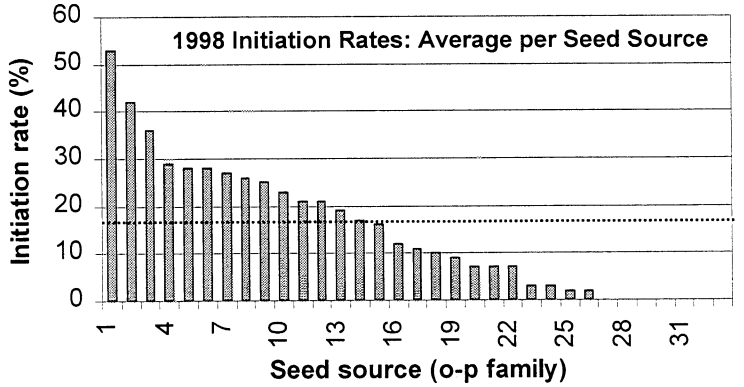
Variation in Initiation and in the Number of Zygotic Embryos per Seed

John MacKay, Heidi Schindler,
Christina Perfetti, Yalin Zhang, Gerald Pullman

Background

Initiation Rates are Variable

- Developmental stage
- Seed source (Genotypes)
- Others (storage, etc.)



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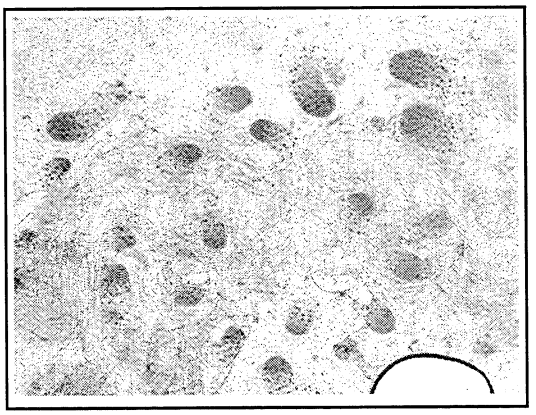
Genetic Variation of Initiation

- Genetic control of initiation demonstrated in spruce (Park et al, 1998)
  - Mature seed: Additive genetic control
  - Immature seed: Dominance and additive genetic control
- Individual pine trees produce seeds with either low, moderate or high initiation rates (Becwar and Chesik, 1994).
- Seed sources with higher initiation rates have a greater number of zygotic embryos per seed (Becwar *et al.*,1997) - Mother tree genotype.

Number of Zygotic Embryos per Seed

- Polyembryony; multiple zygotic embryos per seed:
  - Simple polyembryony: Several fertilizations
  - Cleavage polyembryony: division of early embryos
- The total number of embryos (post-cleavage embryos) per seed is highly variable.



Variation in the Number of Embryos per Seed

Summary of Findings

- **Number of embryos per seed (** post cleavage) was highly variable, ranging from 2 to 20
- **The mother-tree (o-p family)** was a highly significant source of variation in all experiments. The mean number of embryos for each mother-tree ranged from 4.4 ± 1.0 to 12.2 ± 0.95.
- **Site.** The site or geographic region in which the seed were produced had a small effect on the number of embryos per seed, but significant variation was observed in some cases.

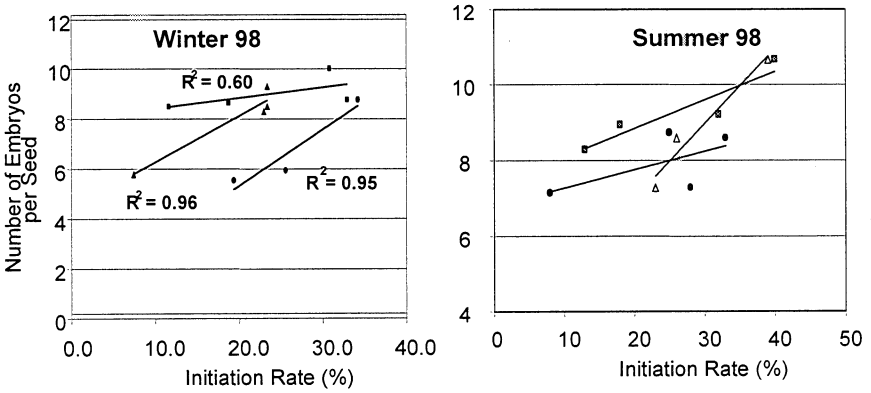
Variation in the Number of Embryos per Seed

Summary of Findings

- **Position of the cone in the tree crown.** Trend toward more embryos per seed in the upper crown and fewer embryos per seed in the lower crown (statistically significant at p= 0.04).
- **Developmental stage** affected the number of embryos per seed which declined gradually initially and more sharply when the dominant embryo reached stage 8 to stage 9 (Figure 2).
- **Within and among cone variability.** The number of embryos in each immature seed varies considerably within a cone, but the average number of embryos per seed varies much less among cones from the same tree.

Number of Embryos and Initiation

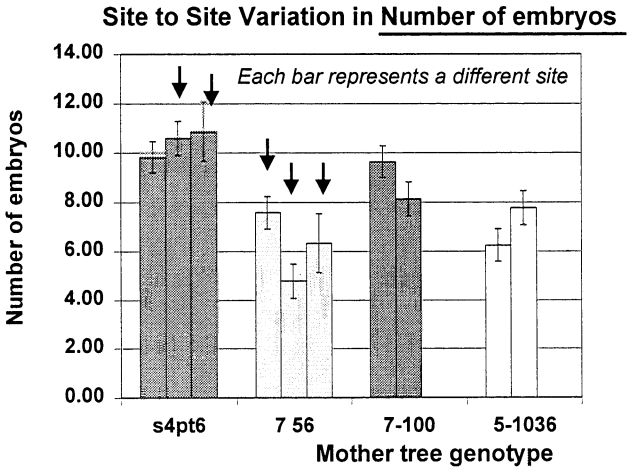
Correlation between the Number of Embryos per seed and Initiation rates



Number of Embryos and Initiation

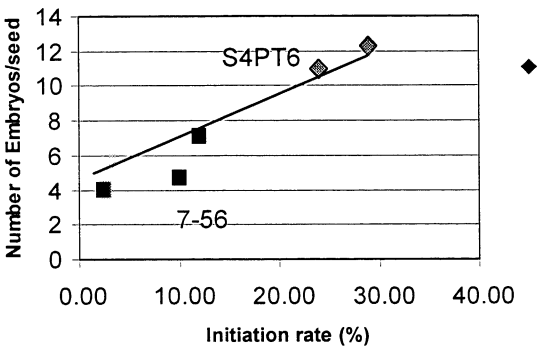
- **Is there a biological basis for the correlation between number of embryos per seed and initiation?**
- **Why is the number of embryos per seed important for initiation?**

Within Family Variation- Number of Embryos



Within family variation - Initiation

Effect of number of embryos on initiation with seed collected from different sites



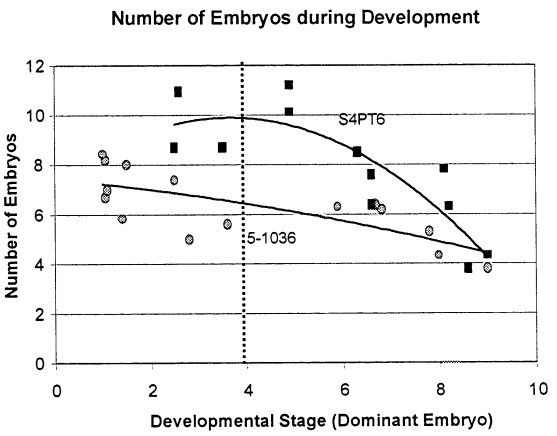
- Site to site variation in initiation is consistent with the variation in the number of embryos per seed.

Role of the Number of Embryos

- Is the number of embryos per seed a driver of initiation?
- Do more embryos simply increase the probability of initiation?
- Does a greater number of embryos indicate an increased potential to proliferate?

Role of the Number of Embryos

- Number of embryos per seed decreases in late development
- Development of subordinate embryos is poorly characterized



Future directions

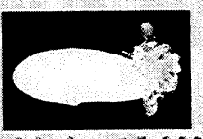
- What controls is the number of embryos
  - Simple polyembryony
  - Cleavage polyembryony
- Genetic control (inheritance) of initiation and number of embryos per seed.
- Is the number of embryos per seed a marker for Initiation Potential?
- Grant proposal activity, Tip3 (State of GA), Agenda 2020.

Survival of Initiated Cultures after Direct Transfer to Liquid Media: The Effect of Genotype, Sugar and Auxin Type

Gary Peter
Teresa Vales

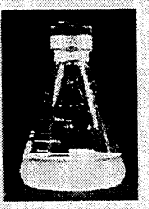
IPST

Efficiency of Somatic Embryogenesis



Initiation 15-30%

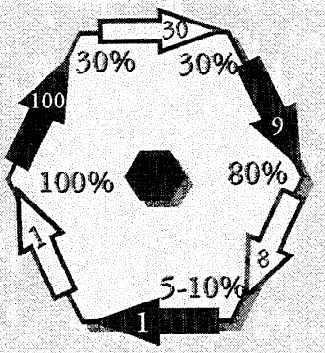
Conversion 80-100%



Multiplication 100%

Germination 0-50%

Maturation 5-90%



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OBJECTIVES

- Test whether direct transfer to liquid culture improves capture of genotypes
- Test the effect of media shock with maltose, NAA and liquid initiation media
- Determine the 1/2 sib family/genotype effects
- Develop hypotheses for why many initiated cultures do not establish liquid cultures

Materials & Methods

- Genotypes - 100 from 7 - 1/2 sib families
- Media -
  - Initiation media 889
  - Multiplication Media 16, Media 16 with maltose substituted for sucrose, Media 16 with NAA substituted for 2,4-D
- 9:1 ratio of media 16: starting mass when possible; otherwise 0.5 ml minimum volume

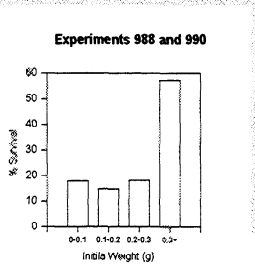
Experimental Procedure & Design

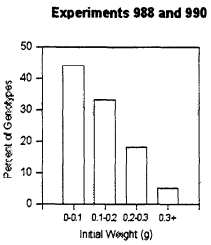
- Procedure
  - Remove somatic/zygotic embryo mass, leaving female gametophyte behind
  - Measure mass of initiate
  - Transfer into appropriate volume of multiplication media
  - Score for growth after 6 weeks
- Design
  - 25 initiates were chosen randomly and transferred into each of the four media; attempts were made to control for starting mass

Media	Sugar	Auxin Type
16	Sucrose	2,4-D
16 -NAA	Sucrose	NAA
16 – maltose	Maltose	2,4-D
943	Maltose/ Inositol	NAA

Summary of Results

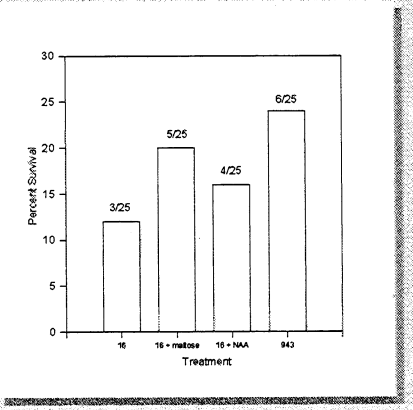
- Overall rate of survival after direct transfer 18%
- High correlation between starting mass and success of transfer (> 0.3g - 55%)
- Most initiates (~70%) do not reach adequate mass within 9-12 weeks on initiation media





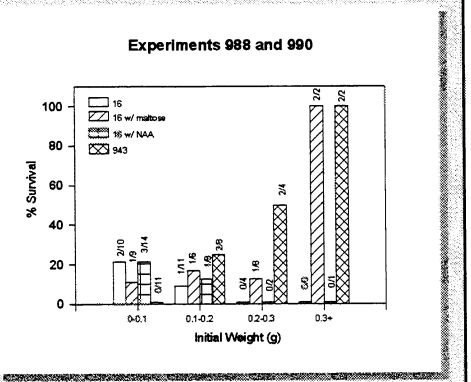
Effect of Media Composition

- Liquid initiation media overall highest
- Small increases with maltose and NAA



Starting Mass vs. Media Composition

- Liquid initiation media best with larger masses, not effective with smaller masses
- Media 16's effective across all weight classes



Effect of 1/2 Sib Family/Genotype

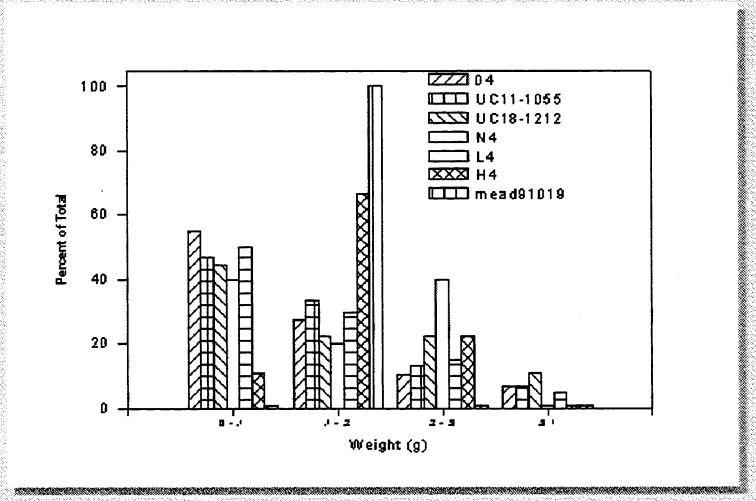
Genotype	% Survival	#Survive/ Total #	# survived/ # Tested 16	# survived/ # Tested 16-malt	# survived/ # Tested 16-NAA	# survived/ # Tested 943
UC 11-1055	6.7	1/15	0/3	0/5	0/3	¼
UC 18-1212	11.1	1/9	0/2	0/2	0/3	½
WV H4	11.1	1/9	0/2	0/1	1/3	0/3
WV L4	10.0	2/20	0/5	1/5	0/5	1/5
WV N4	13.3	2/15	0/4	¼	0/3	¼
WV O4	37.9	11/29	3/8	3/7	3/8	2/6

1/2 Sib Family/Genotype Effects on Initiate Survival in Liquid Multiplication Media 16

- Significant genotype effects are observed for culture survival
- Genotype effects on initiation are different than for culture survival

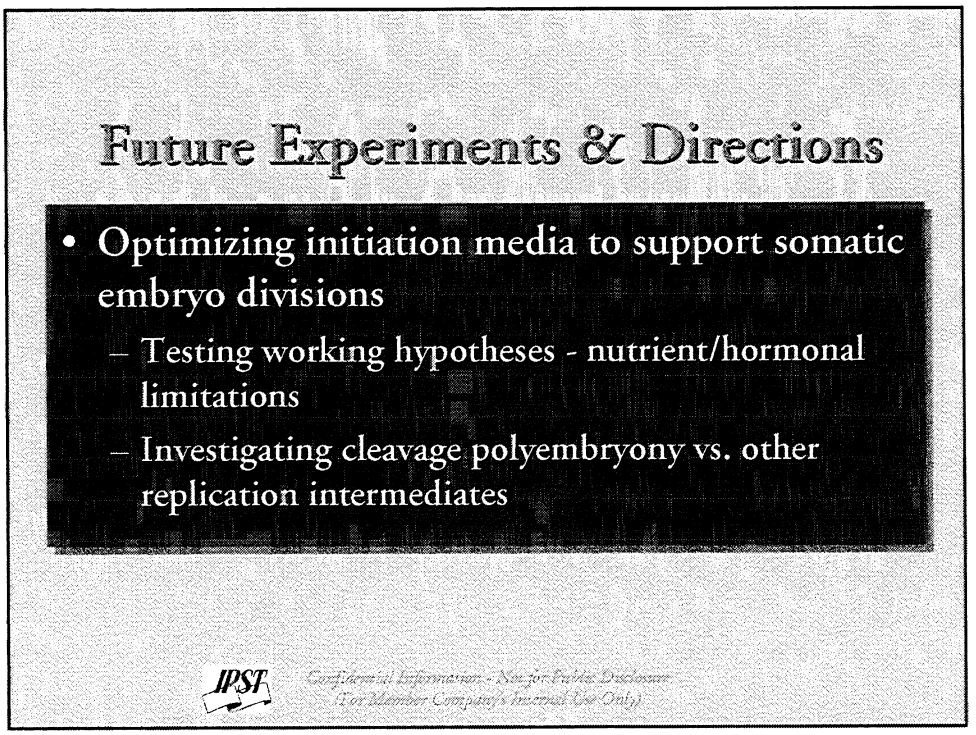
Genotype	% Survival	#Survive/ Total#	# survived/ #Tested 16	Initiation Rate (%)
Spring PAC				
UC 1-1055	6.7	1/15	0/3	16.2
UC 8-1212	11.1	1/9	0/2	21.1
WV H4	11.1	1/9	0/2	
WV L4	10.0	2/20	0/5	
WV N4	13.3	2/15	0/4	41.7
WV O4	37.9	11/29	3/8	28.4
M 91019	0	0/3	0/1	
Fall PAC				
UC5-1507	66.7	2/3	2/3	
UC5-1036	33.3	5/15	5/15	
UC11-1057	83.3	5/6	5/6	
UC11-1178	0	0/2	0/2	
UC18-1212	15.1	11/73	11/73	
WV J3	0	0/2	0/2	
S4PT6	0	0/1	0/1	

1/2 Sib Family & Initiate Mass



Conclusions

- Direct transfer into liquid is efficient for large masses (≥ 0.15g)
- Conceptually separate initiation into 3 phases
  - extrusion
  - somatic embryo differentiation
  - multiplication - cleavage polyembryony?


Future Experiments & Directions
• Optimizing initiation media to support somatic embryo divisions
– Testing working hypotheses - nutrient/hormonal limitations
– Investigating cleavage polyembryony vs. other replication intermediates
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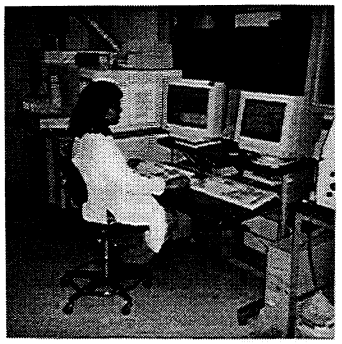
ELEMENTAL ANALYSIS OF ZYGOTIC FEMALE GAMETOPHYTE & EMBRYO TISSUES



Gerald Pullman
Mike Buchanan
Paul Montello
Yolanda Powell
Xiaorong Feng

Elemental Analyses for Media Improvement - Approach

- **Determine zygotic tissue elemental composition**
- **Determine somatic embryo elemental composition**
- **Modify medium based on zygotic target**
- **Analyze resulting somatic embryos for elemental composition, repeat cycle**
- **Determine zygotic tissue composition over developmental sequence**

Comparison of elemental compositions for zygotic female gametophyte, zygotic embryo, and somatic embryo tissues with along with the ratio for each element found in somatic / zygotic embryos.

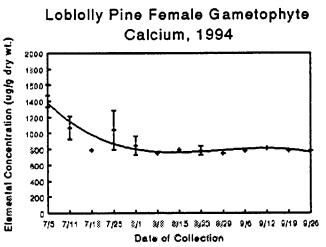
Metal	*Mn*	*Fe*	*Ni*	*Cu*	*Zn*	*B*	*P*	*S*	*Na*	*Mg*	*K*	*Ca*
Gametophyte	227	71	3	20	149	19	12215	5388	4	5132	9112	256
zygotics	81	231	1.8	27.4	130	4.5	16246	2466	6.9	7609	12075	182
Somatics	54	49		2.8	124	78	7449	2713	1315	3820	22204	551
Ratio SE/ZE	0.66	0.21	0	0.10	0.95	17.2	0.46	1.1	191	0.50	1.8	3.0

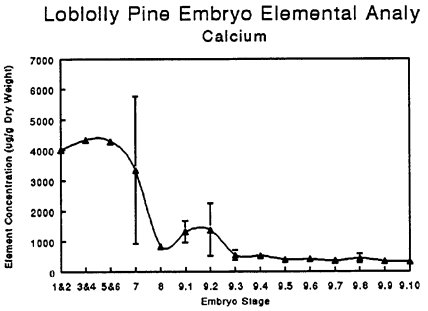
Hypothesis: Changes in tissue culture media to more closely match zygotic embryo elemental content will improve embryo quantity and quality.

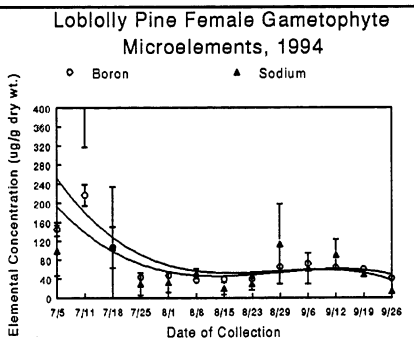
Elemental Analysis Summary

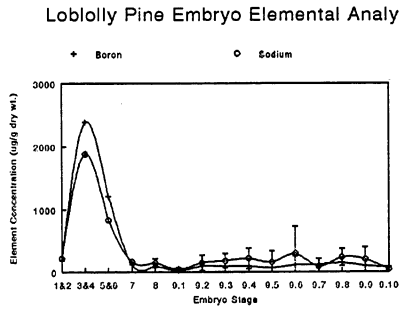
(somatic/zygotic)

- Boron (1720%)
- Calcium (300%)
- Copper (10%)
- Iron (21%)
- Phosphorous (46%)
- Magnesium (50%)
- Potassium (180%
- Manganese (.66%)









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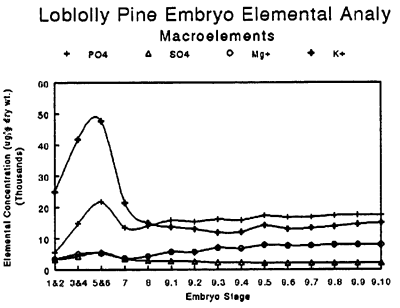

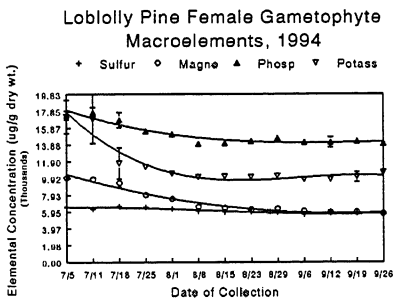

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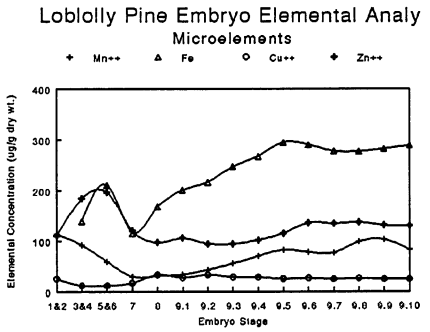

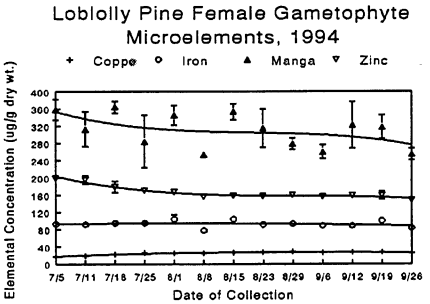

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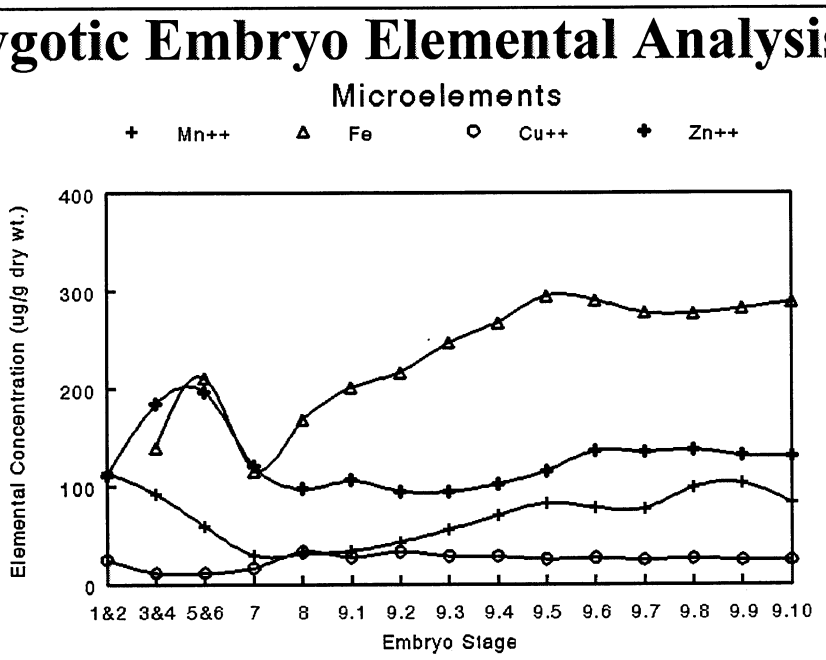

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Table 16. Elemental analysis (mg/Kg) for oven-dried somatic embryos of clone 333.

Medium	Mn	Fe	Cu	Zn	B	P	S	Na	Mg	K	Ca
240 = 1x B, 1x Ca, 1x Fe	37.3	56.4	6.2	95.9	41.4	8989	3535	1172	4369	27088	7395
705 = ½x B, ½ Ca, 1x Fe	28.3	36.5	3.6	83.6	<5.3	7594	3551	1743	3976	26659	3338
751 = ¾x B, ½x Ca, 1x Fe	51.4	59.8	6.3	82.7	20.4	9637	3632	1088	4928	26310	4032
759 = ½x B, ½x Ca, 1.5x Fe	35.6	81.4	4.7	85.7	16.4	8576	3777	1336	3999	29564	3604

Conclusions

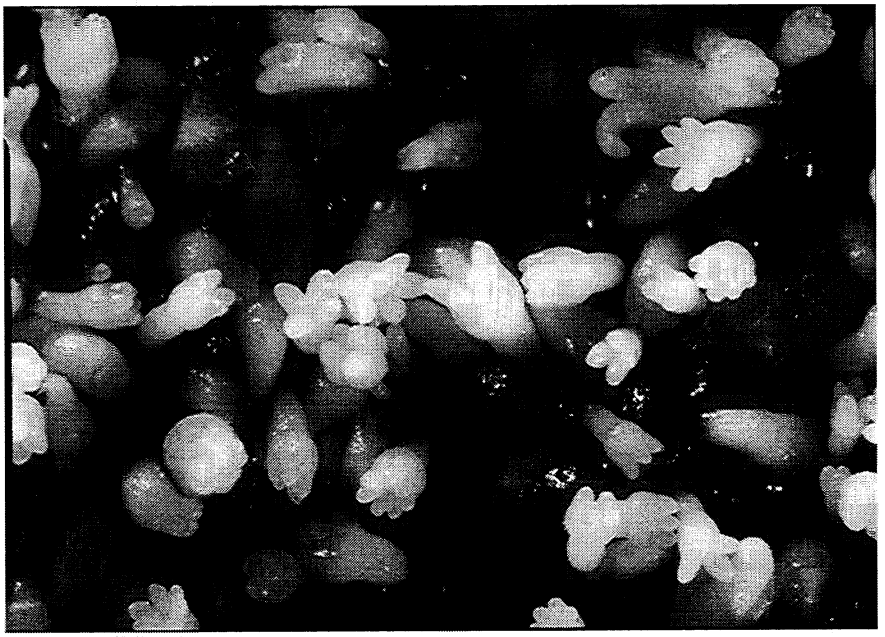
- **Increasing Iron in the development & maturation media allows statistically significant increases in embryo yield.**
- **These increases do not give significant increases in dry weight.**

Conclusions

- **After considering all the data, we have settled on 3x Iron in our maturation & development media.**
- **The three fold increase in iron coincides well with the increase found in zygotic embryos through development.**

Spring 1999 Summary of IPST loblolly pine embryogenesis: past, current, & target performance for major process steps.

TISSUE CULTURE STEP	1993	1994	1995	1996	1997	1998	Target
Embryo Maturation							
Yield Stage 6+ /per ml cells)	<1	10+	10+	10.6	142	190	25+ High Quality
Stage (quality)	7	8	8	6-8	8-9.1	8-9.1	9.4
#Genotypes	~3	~5	~5	14/25 (56%)	15/18 (83%)	7/12 (58%)	50% liq cultures
Germination (shoot /root)	0%	30%	30%	0-33%			75% of embryos
Germination - Genotypes		1	1	4/25 (16%)			50% liq cultures



Optimizing the Percent of Embryos that Develop: The Role of Plating Density and ABA Concentration

Gary Peter
Teresa Vales

Objectives

- Develop a method for the maturation of loblolly pine somatic embryos that can be used for a high percentage of genotypes that will result in many high quality embryos that have the functional capability of germinating

Overview

- Section 1: Optimizing embryo plating densities with respect to ABA concentrations
- Section 2: Assessing the ratio of cell density to ABA concentration and the reliability of using this ratio to optimize embryo development
- Section 3: Using the weight of cells plated to assess the reliability of our plating methods

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Optimizing Embryo Plating Densities With Respect To ABA Concentration In The Media

- Previous Findings
  - 1ml settled cell plating density is not optimal due to a combination of nutritional and ABA limitations
  - These limitations can be overcome by decreasing the embryo plating density
  - Embryo development can be limited by ABA deficiency as well as excess ABA

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Optimizing Embryo Plating Densities With Respect To ABA Concentration In The Media

- Hypotheses
  - Similar trends will be seen using alternative genotypes
  - Can provide more information about the existence of a ratio of cell density to ABA that can predict high percent development and whether this ratio is genotype-specific

Methods

- Settled cells from genotype 455 were diluted 1/20 with media 16
- Varying amounts of the dilution were added to a vacuum filter apparatus to obtain the following settled cell amounts per plate: 0.125, 0.25, 0.33, 0.5, and 1ml
- The black filter papers were transferred to plates with media 752 amended to contain varying amounts of ABA (19.6-98 uM)

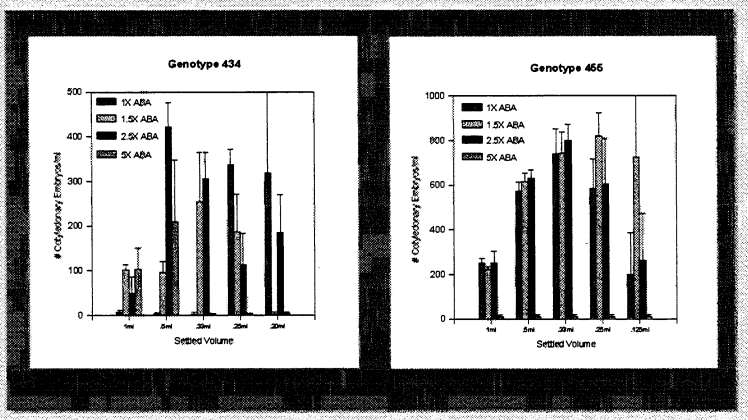
Methods

- Plates were wrapped in parafilm and placed in the dark
- Embryos were subcultured monthly
- At the end of two months, the number of cotyledonary embryos was counted
- The number of cotyledonary embryos per ml was then determined

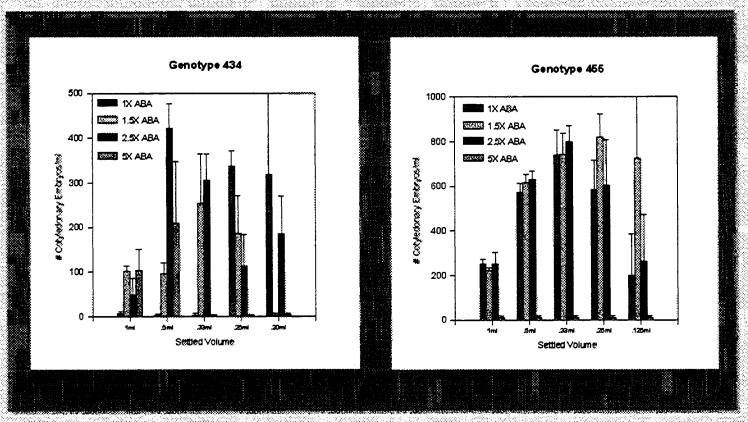
Experimental Design

Plated	ABA
1ml	19.6 uM
1ml	29.4 uM
1ml	49.0 uM
1ml	98.0 uM
0.5ml	19.6 uM
0.5ml	29.4 uM
0.5ml	49.0 uM
0.5ml	98.0 uM
0.33ml	19.6 uM
0.33ml	29.4 uM
0.33ml	49.0 uM
0.33ml	98.0 uM
0.25ml	19.6 uM
0.25ml	29.4 uM
0.25ml	49.0 uM
0.25ml	98.0 uM
.125ml	19.6 uM
.125ml	29.4 uM
.125ml	49.0 uM
.125ml	98.0 uM

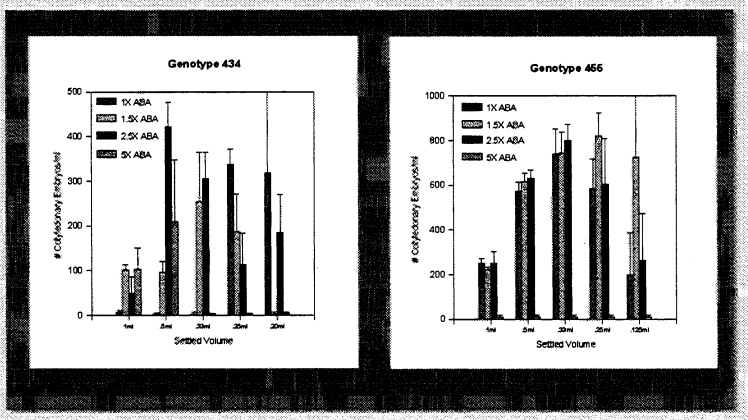
Embryo Development Can Be Limited By High Concentrations of ABA



The Number of Embryos That Developed At 1ml is Lower Than At Reduced Cell Densities



The Number of Embryos That Develop at Each Cell Density Changes With The Concentration of ABA in the Media



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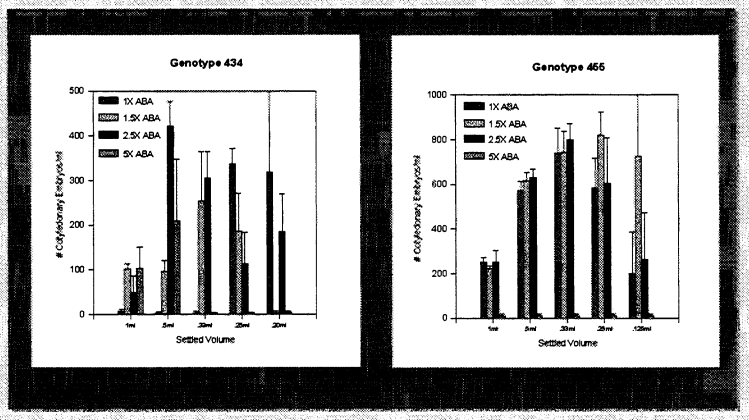
Data Suggests the Existence of a Ratio of Cell Density to [ABA] in the Media That Can Be Used To Predict High Rates of Embryo Development



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Assessing the Ratio of Cell Density to ABA Concentration and the Reliability of Using This Ratio to Optimize Embryo Development

- Hypotheses
  - Can achieve a high percentage of embryo development if we maintain a particular ratio of cells plated to ABA in the media
  - Optimizing this ratio for each genotype may lead to increased embryo quality

IPST

Is the ratio of initial embryo number plated to the concentration of ABA in the media a reliable predictor of development?

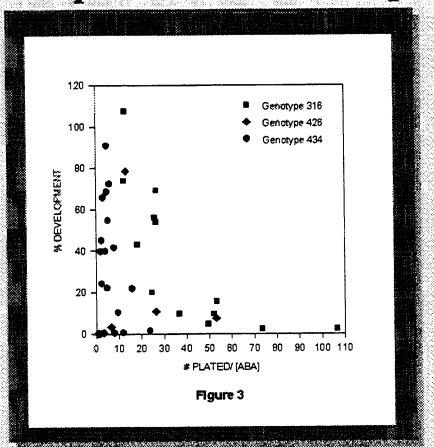

Figure 3

IPST

Is the ratio of settled volume to the concentration of ABA in the media a reliable predictor of embryo development?

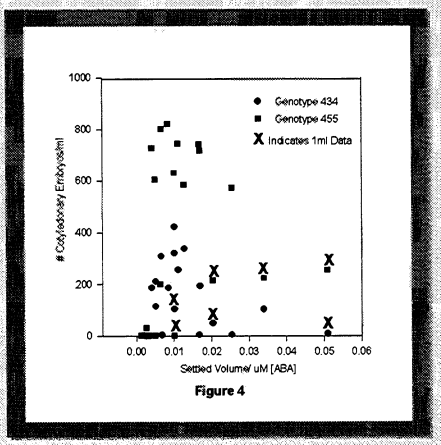

Figure 4

Conclusions

- An upper limit for the ratio as well as an optimal ratio is suggested
- The development occurring around the optimal ratio is a mixture of high and low

Reasons Why Cell Weight/[ABA] Would Be a Better Predictor of High Rates of Embryo Development

- Weight would consider the presence of non-embryogenic cell that would in addition to embryos, take in nutrients
- Weight should be more accurate and consistent from week to week than settled volume

Using the Weight of Cells Plated to Assess the Reliability of Our Plating Method

- Hypotheses
  - Error between replicate plates in the number of cotyledonary embryos produced per plate is largely due to the method used in transferring cells to the black filter paper
  - By weighing the cells applied, the error as well as improvement attempts can be assessed

Using the Weight of Cells Plated to Assess the Reliability of Our Plating Method

- Hypotheses
  - Using a spinner flask to mix the diluted cells can decrease the percent error between replicate plates
  - Using a more viscous media such as liquid 752 to dilute the cells can decrease the error between replicate plates

Method

- Settled cells were diluted 1/20 with media
- A black filter paper was wetted, placed on the vacuum apparatus, then weighed
- Cells from the dilution were added to the black filter paper via the vacuum filter apparatus
- The weight of the cells was obtained
- Percent error between replicates was determined

Using the Weight of Cells Plated to Assess the Reliability of our Plating Methods

Genotype	Amount	Weight	% Error	Comments
426	1ml	0.2123	13.0	vacuum applied
426	.5ml	0.1013	5.75	vacuum applied
426	.33ml	0.0863	20.5	vacuum applied
426	.25ml	0.0702	19.7	vacuum applied
426	.125ml	0.0543	28.3	vacuum applied
479	1ml	0.1383	14.1	directly applied
479	1ml	0.1447	7.46	vacuum applied
479	.5ml	0.0659	7.51	vacuum applied
479	.33ml	0.0344	32.9	vacuum applied
479	.25ml	0.0265	41.8	vacuum applied
479	.125ml	0.0188	24.0	vacuum applied
434	1ml	0.1465	8.12	directly applied
434	1ml	0.1668	9.34	spinner flask
434	.5ml	0.0752	17.4	spinner flask
434	.33ml	0.0491	18.4	spinner flask
434	.25ml	0.0366	15.7	spinner flask
434	.125ml	0.0168	28.2	spinner flask
452	1ml	0.1347	16.1	directly applied
452	1ml	0.1416	6.16	spinner flask
452	.5ml	0.0671	19.6	spinner flask
452	.33ml	0.0409	21.6	spinner flask
452	.25ml	0.0388	38.8	spinner flask
452	.125ml	0.021	44.4	spinner flask

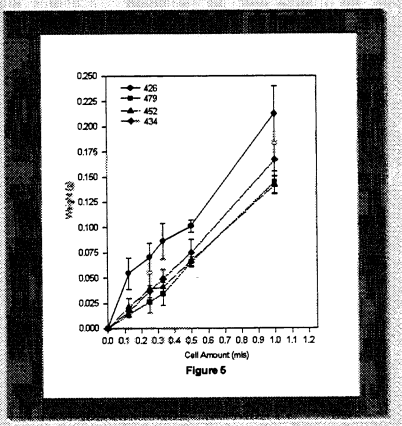

Figure 6

Using the Weight of Cells Plated to Assess the Reliability of our Plating Methods

Genotype	Amount	Weight	% Error	Comments
482	1ml	0.1763	6.30	media 16
482	.5ml	0.0755	6.89	media 16
482	.25ml	0.0383	13.6	media 16
482	1ml	0.1236	4.25	media 752
482	.5ml	0.0635	6.70	media 752
482	.25ml	0.0455	12.2	media 752
461	1ml	0.2187	28.8	media 16
461	1ml	0.0796	23.0	media 752

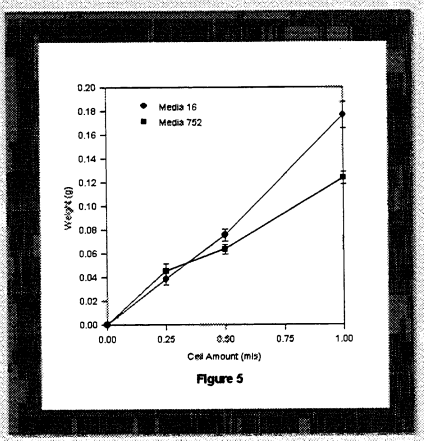

Figure 5

Conclusions

- Error between replicates can be improved by vacuum application
- Error is not improved by better mixing of the diluted cells or by increasing the viscosity of the diluting fluid
- Error can be correlated with the size of the tissue particles in solution with more error associated with the larger tissue pieces
- Weighing the cells plated is a good way to assess the error associated with plating cells

Conclusions

- Error between replicate plates is most likely due to the method of transferring the cells
- Weighing the cells is a good way to assess the error associated with plating cells

FIELD ESTABLISHMENT OF SOMATIC EMBRYO DERIVED LOBLOLLY PINE SEEDLINGS

Gerald Pullman
Paul Montello
Mike Cunningham

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IPST Somatic Seedling				UC Seedling	
Tree	HT	Tree	HT	Tree	HT
1	5.2	21	5.2	1	8.8
2	6.3	22	3.0	2	9.7
3	4.6	23	5.4	3	10.2
4	3.3	24	6.8	4	8.2
5	5.3	25	5.5	5	7.3
6	5.0	26	5.6	6	7.8
7	3.4	27	5.5	7	10.0
8	5.3	28	filler	8	7.4
9	5.7	29	6.6	9	7.7
10	5.8	30	5.9	10	7.7
11	5.8	31	4.9	11	9.1
12	6.6	32	4.9	12	8.2
13	6.5	33	4.3	13	7.0
14	5.0	34	4.8	14	9.3
15	4.8	35	5.0	15	8.0
16	5.6	36	5.2	16	11.0
17	5.6			17	10.0
18	5.5			18	8.0
19	4.4			19	8.3
20	35			20	7.3
Average		5.2 ft.			8.6 ft.

Three Additional Genotypes Growing in the Greenhouse

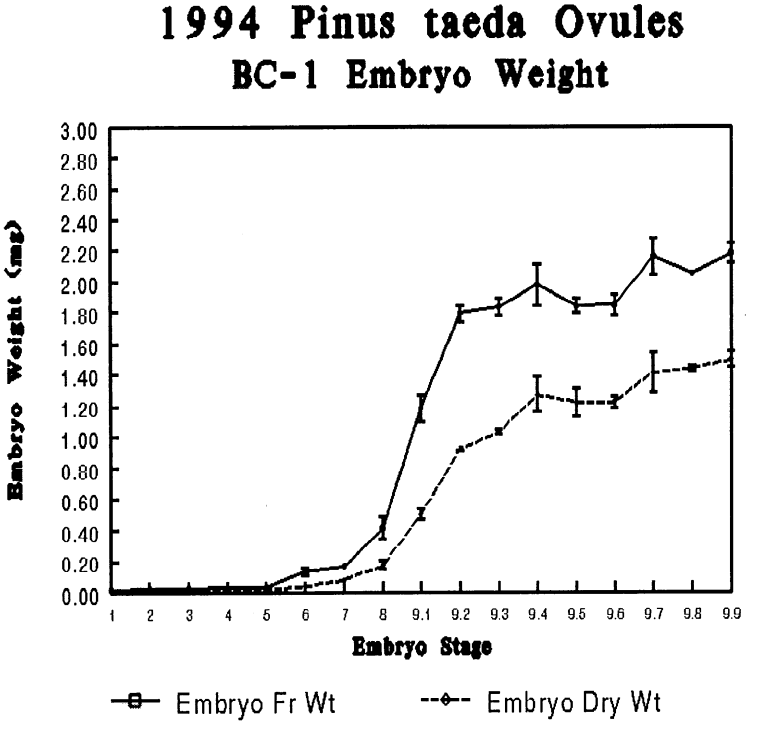
Spring 1999 Summary of IPST loblolly pine embryogenesis: past, current, & target performance for major process steps.

TISSUE CULTURE STEP	1993	1994	1995	1996	1997	1998	Target
Initiation 505 (18 mother trees)	<1%	<1%	16%	7.4%	8.5%[4]		35%
889 (32 mother trees)						**17.9%**[4]	
Maintenance							
Survival on gelled media							
Short-term (<6 months)				20%	42%L	**16-24%L**	50%
Long-term (>6 months)	83%[1]			G			
Growth (G=gelled, L=liquid)	L2.5/wk	L2.5/wk	L2.5/wk				OK
Cryogenic Storage				L2.5/wk			80% liq
				74%			Cultures
Liquid Culture Embryo Quality	25%[2]			64%[3]	50-90%[5]	50-90%[5]	OK
Embryo Maturation							
Yield Stage 6+ /per ml cells)	<1	10+	10+	10.6	142	**190**	25+ High Quality
Stage (quality)	7	8	8	6-8	8-9.1	8-9.1	9.4
#Genotypes	~3	~5	~5	14/25	15/18	7/12	50% liq cultures
				(56%)	(83%)	(58%)	
Germination (shoot /root)	0%	30%	30%	0-33%			75% of embryos
Germination - Genotypes		1	1	4/25 (16%)			50% liq cultures
Acclimation -% of embryos			94%				80% of germinants
Acclimation - Genotypes			1	2	4	4	50% of L cultures

[1]/ Already existing cultures, previous survival 6 months or longer. [2]/ Percent of cultures which produce early-stage embryos of stage 2 or better in liquid medium 16, starting cultures maintained in maintenance medium longer than 1 year. [3]/ Percent of cultures which produce early-stage embryos of stage 2 or better in liquid medium 16, starting cultures were recently initiated with growth in initiation or maintenance medium less than 3 months. **[4]/ Data in bold format represent changes within the past six months. [5]/ The % of early stage embryos that develop into cotyledonary embryos.**

Somatic Embryo Quality - Where Are We?

- Morphologically - stage 8-9.1
- Dry weight - stage 8- 9.1
- Development similar to early August
- Molecular biology- bands missing 9.1-end
- Germination - stage 7-8

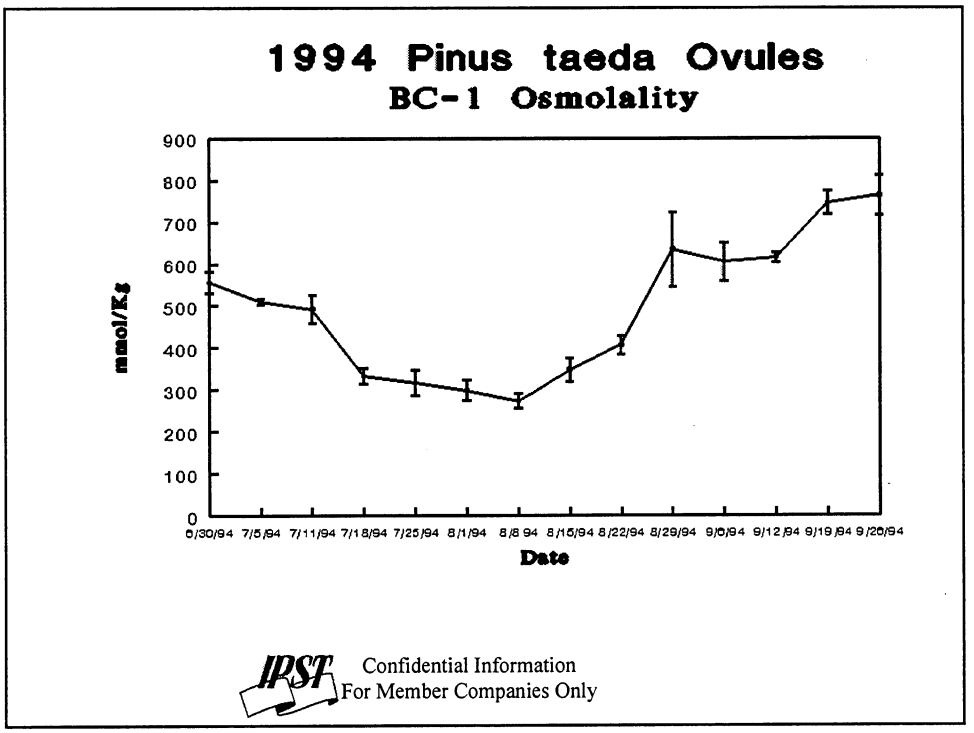


ANALYSIS OF FREE AMINO ACIDS IN ZYGOTIC FEMALE GAMETOPHYTE AND EMBRYO TISSUES

Gerald Pullman
Yalin Zhang
Christine Estes
Mike Buchanan

INTRODUCTION

- Free amino acids are one of the groups of compounds which contribute to water potential.
- Free amino acids are precursors for nitrogen compounds and proteins


1994 Pinus taeda Ovules
BC-1 Osmolality
mmol/Kg
900
800
700
600
500
400
300
200
100
0
6/30/94 7/5/94 7/11/94 7/18/94 7/25/94 8/1/94 8/8 94 8/15/94 8/22/94 8/29/94 9/5/94 9/12/94 9/19/94 9/26/94
Date
IPST
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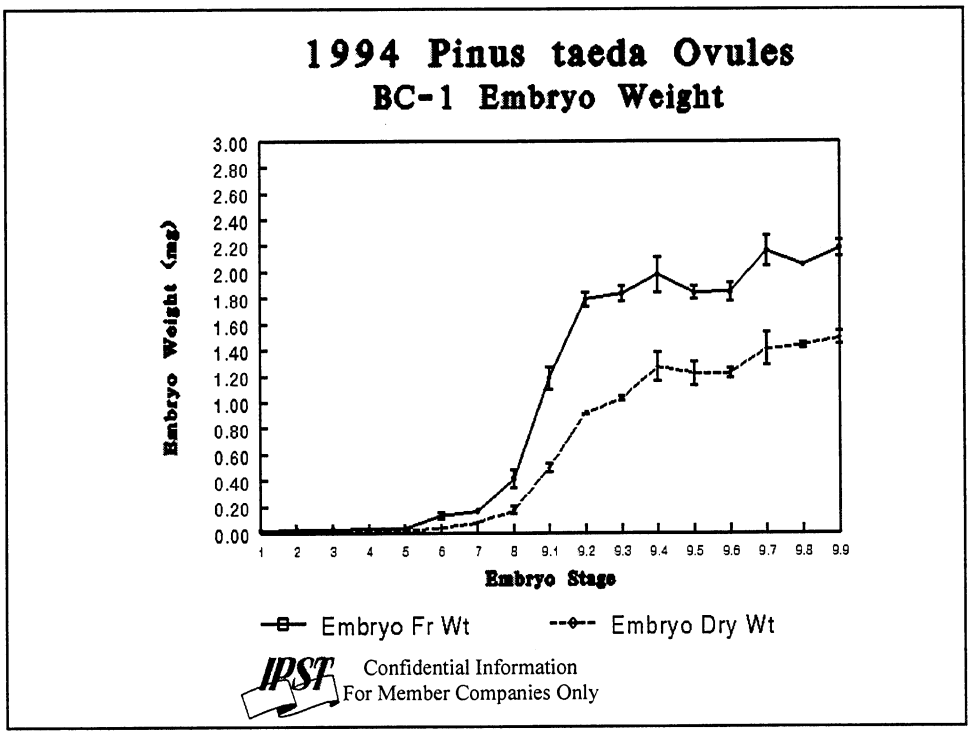
1994 Pinus taeda Ovules
BC-1 Embryo Weight
Embryo Weight (mg)
3.00
2.80
2.60
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1 2 3 4 5 6 7 8 9.1 9.2 9.3 9.4 9.5 9.6 9.7 9.8 9.9
Embryo Stage
Embryo Fr Wt
Embryo Dry Wt
IPST
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General Method

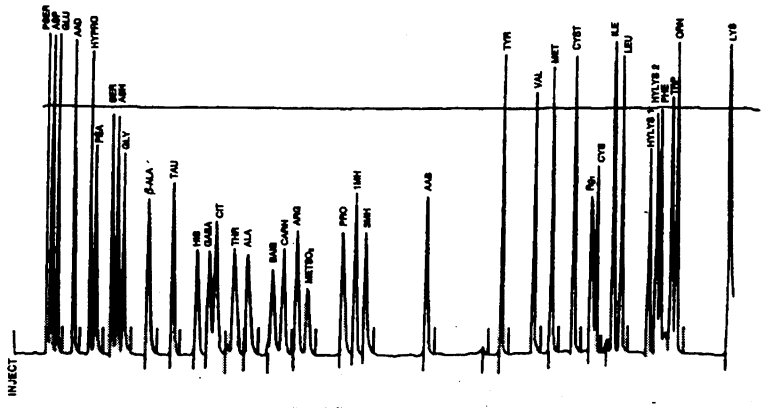
- Embryos staged, collected & stored at -70 C
- Tissue was freeze-dried, weighed
- Extracted free amino acids in 80% ethanol
- Extract filtered by 10K MW cut off
- Free amino acids derivatized
- HPLC Analysis

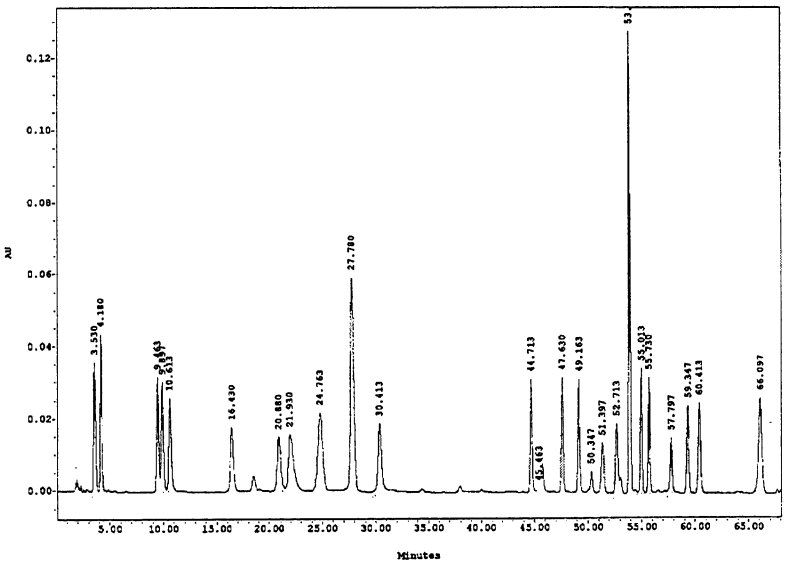
EQUIPMENT

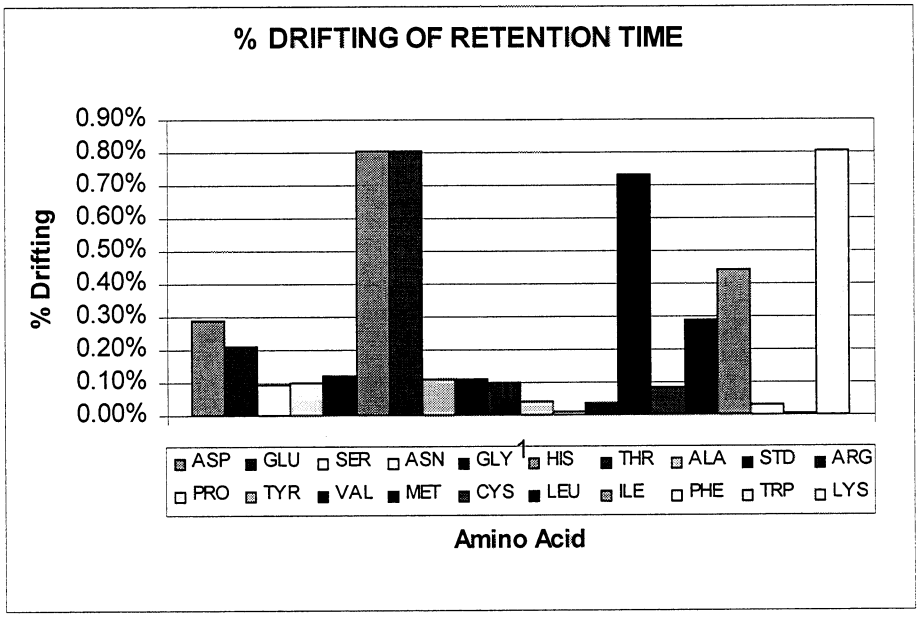
- Waters 600 HPLC System
- Millennium Software Package

Problems and Modifications

- Problem--Unrepeatable results
- Modifications
  - In-line Degasser
  - Injection Loop
  - New C-18 Pico-Tag Column
- Preparation of Amino Acid standards







19 Basic Amino Acids

- ASP Aspartic Acid
- GLU Glutamic Acid
- SER Serine.
- ASN Asparagine
- GLY Glycine
- HIS Histidine
- THR Threonine
- ALA Alanine
- ARG Arginine
- PRO Proline
- TYR Tyrosine
- VAL Valine
- MET Methionine
- CYS Cystine
- LEU Isoleucine
- ILE Leucine
- PHE Phenylalanine
- TRP Tyrptophan
- LYS Lysine

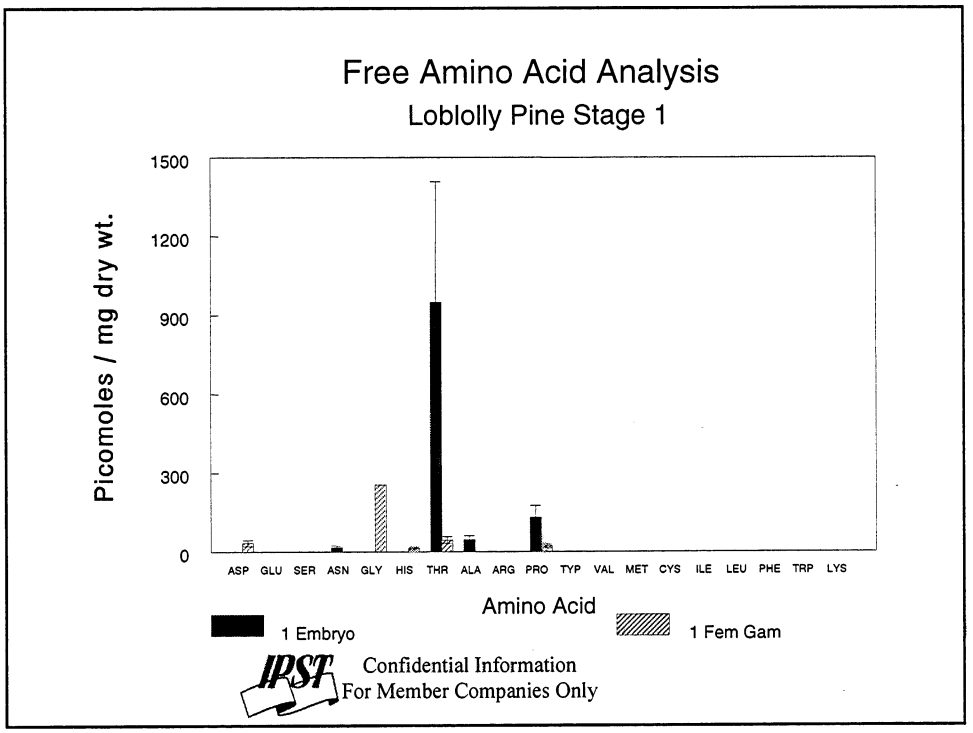
Glutamine Not measured

What We Improved after last PAC Meeting

Auto Sampler

	Free Amino Acids in Embryo Tissues (Stages 1-9.10)									
FREE AA	Stage 1	SE	Stage 4	SE	Stage 8	SE	Stage 9.3	SE	Stage 9.1	SE
	2 reps		1 rep		3 reps		3 reps		3 reps	
	Embryo		Embryo		Embryo		Embryo		Embryo	
ASP			1190.15		180.05	23.18	287.47	115.44	15.18	0.93
GLU			1375.50		1438.93	480.16	514.17	85.53	95.47	4.88
SER			498.36		355.32	84.23	110.92	0.55	23.81	1.76
ASN	16.33	5.77	332.13		487.02	110.35	85.75	44.84	73.98	7.37
GLY			66.61		145.77	44.55			11.31	1.28
HIS					90.91	20.36			7.03	1.38
THR	948.05	460.05	206.81		139.19	56.93	164.27	38.20	11.50	0.64
ALA	45.22	15.99	916.48		799.50	176.53	211.03	49.12	24.67	2.04
ARG					367.50	88.59			66.29	12.70
PRO	129.74	45.87	1879.05		944.83	256.25	424.26	166.43	21.74	2.15
TYR			164.90		96.44	23.46	85.49	29.28	22.04	1.89
VAL			476.59		215.67	48.63	113.17	15.85	23.54	0.99
MET			188.86		71.93	18.24	38.35	3.72	18.40	1.09
CYS			64.34		23.84	5.77	22.75	10.10	1.42	0.13
LEU			664.00		104.31	23.42	198.00	21.32	8.92	0.76
ILE			275.60		152.50	36.83	60.88	11.24	13.20	0.99
PHE			222.80		95.25	21.71	52.07	7.69	10.85	0.68
TRP					31.38	6.48			7.57	0.64
LYS					45.26	11.74			4.07	0.51

	Free Amino Acids in Female Gametophyte Tissues (Stages 1-9.10)									
FREE AA	Stage 1	SE	Stage 4	SE	Stage 8	Stg 8 S	Stage 9.3	SE	Stage 9.1	SE
	2 reps		1 rep	1 rep	3 reps	3 reps	3 reps	3 reps	3 reps	3 reps
ASP	32.63	11.54			66.82	10.43	171.95	46.75	6.32	1.29
GLU					207.57	22.72	592.01	86.60	21.03	4.50
SER					66.57	24.19	91.73	21.39	7.72	0.67
ASN					43.53	15.39	20.13	8.89	36.89	1.19
GLY	254.08				24.07	8.91	18.19	5.95	4.03	0.22
HIS	13.44	4.75			20.83	6.89			1.07	0.87
THR	44.09	13.15			31.28	11.86	199.95	57.42	4.57	0.75
ALA					51.84	27.18	161.03	35.92	8.86	0.37
ARG					426.12	112.34			9.99	4.15
PRO	21.42	7.57			107.48	38.64	351.68	89.49	5.85	0.89
TYR					29.28	11.12	29.63	5.57	7.22	0.89
VAL					53.94	15.67	94.70	22.61	11.94	0.40
MET					33.60	16.33	54.58	13.79	6.09	0.24
CYS					1.02	0.42	38.51	9.36	0.00	0.00
LEU					30.96	11.56	95.48	33.52	7.08	0.35
ILE					57.36	22.97	45.01	11.65	11.16	0.45
PHE					26.20	10.73	32.27	13.32	4.81	0.23
TRP					8.49	2.40			6.42	0.81
LYS					15.68	4.36			0.71	0.58


Free Amino Acid Analysis
Loblolly Pine Stage 1
Picomoles / mg dry wt.
1500
1200
900
600
300
0
ASP GLU SER ASN GLY HIS THR ALA ARG PRO TYP VAL MET CYS ILE LEU PHE TRP LYS
Amino Acid
1 Embryo
1 Fem Gam
IPST
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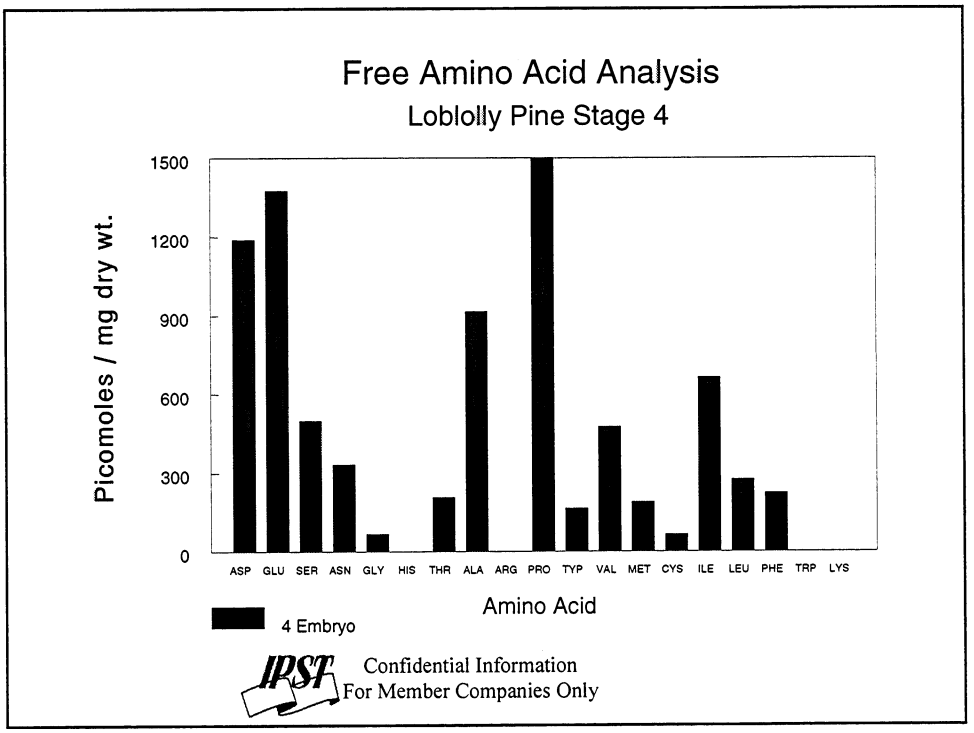
Free Amino Acid Analysis
Loblolly Pine Stage 4
Picomoles / mg dry wt.
1500
1200
900
600
300
0
ASP GLU SER ASN GLY HIS THR ALA ARG PRO TYP VAL MET CYS ILE LEU PHE TRP LYS
Amino Acid
4 Embryo
IPST
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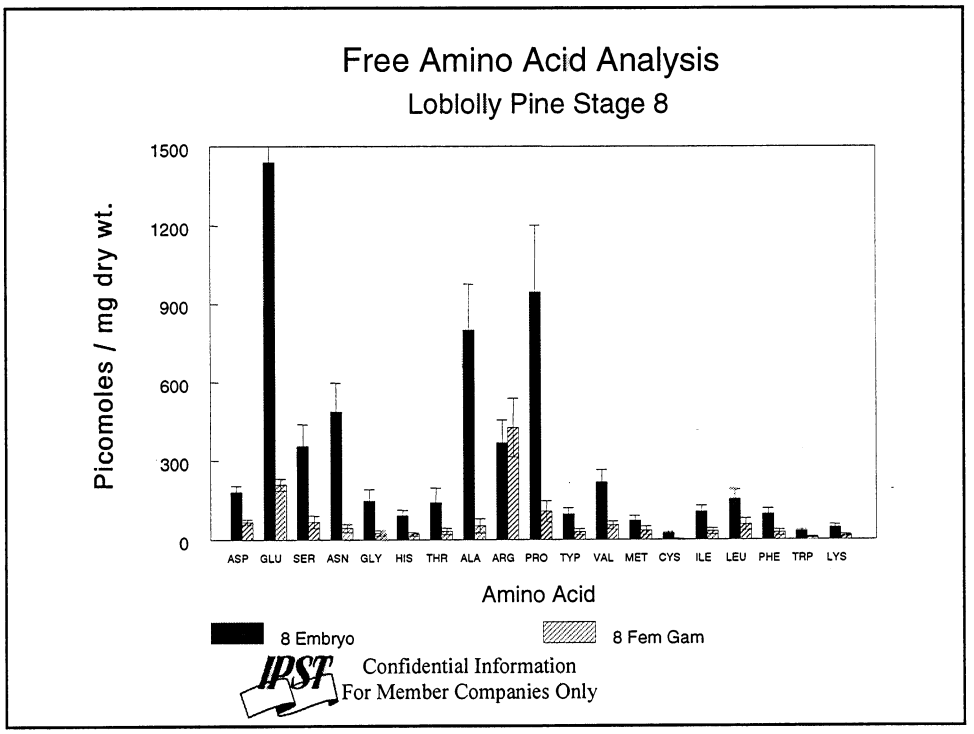
Free Amino Acid Analysis
Loblolly Pine Stage 8
Picomoles / mg dry wt.
1500
1200
900
600
300
0
ASP GLU SER ASN GLY HIS THR ALA ARG PRO TYP VAL MET CYS ILE LEU PHE TRP LYS
Amino Acid
8 Embryo
8 Fem Gam
IPST
Confidential Information
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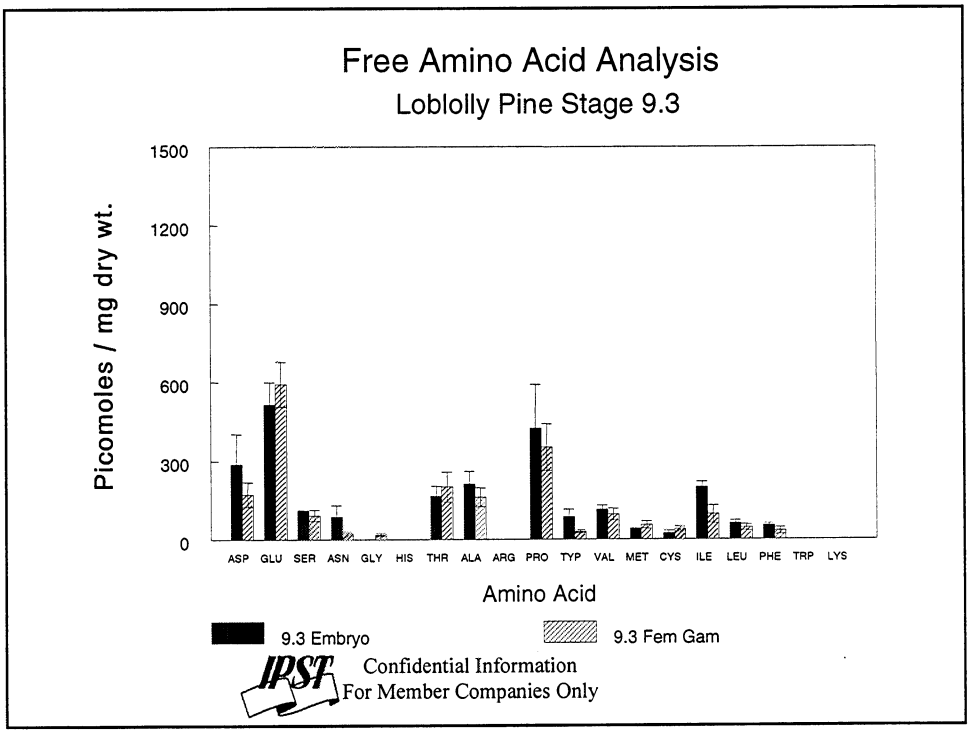
Free Amino Acid Analysis
Loblolly Pine Stage 9.3
Picomoles / mg dry wt.
1500
1200
900
600
300
0
ASP GLU SER ASN GLY HIS THR ALA ARG PRO TYP VAL MET CYS ILE LEU PHE TRP LYS
Amino Acid
9.3 Embryo
9.3 Fem Gam
IPST
Confidential Information
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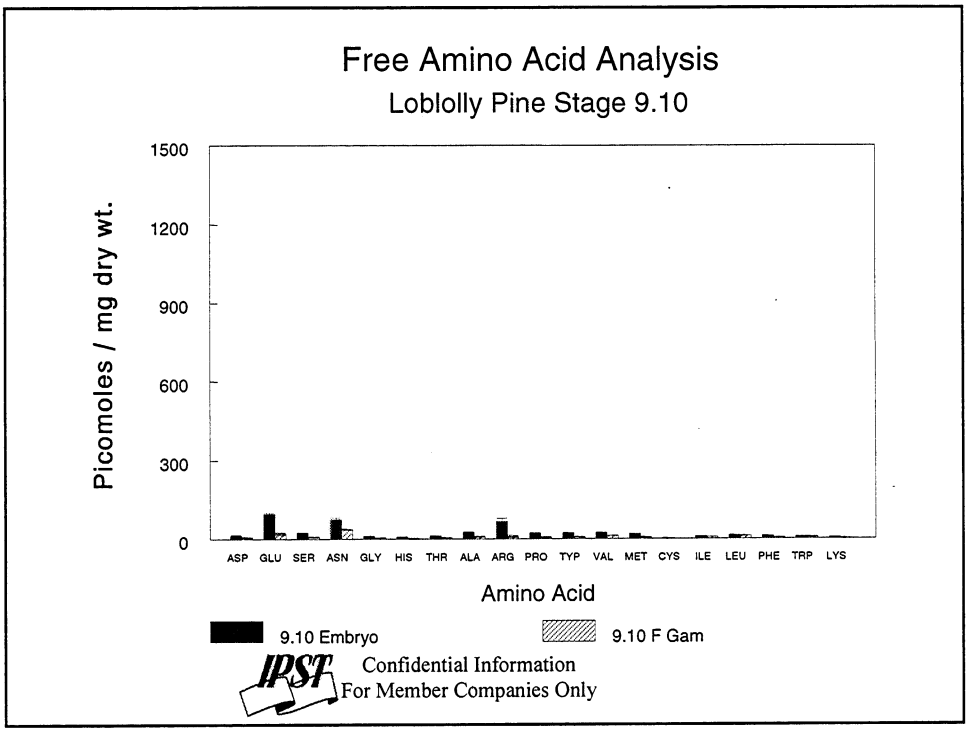
Free Amino Acid Analysis
Loblolly Pine Stage 9.10
Picomoles / mg dry wt.
1500
1200
900
600
300
0
ASP GLU SER ASN GLY HIS THR ALA ARG PRO TYP VAL MET CYS ILE LEU PHE TRP LYS
Amino Acid
9.10 Embryo
9.10 F Gam
IPST
Confidential Information
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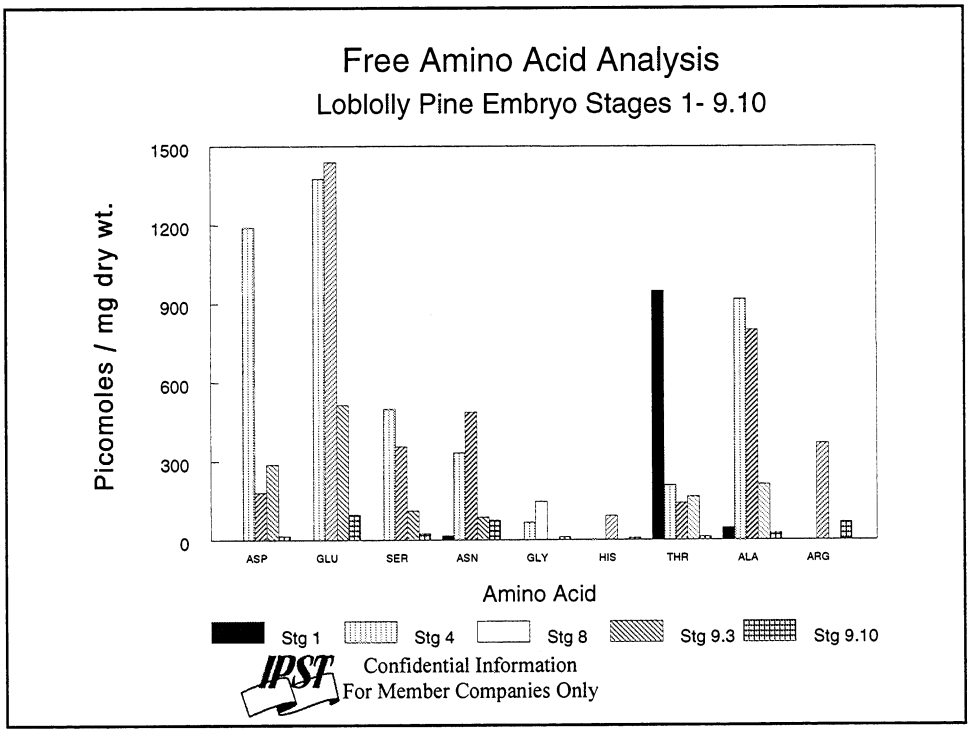
Free Amino Acid Analysis
Loblolly Pine Embryo Stages 1- 9.10
Picomoles / mg dry wt.
1500
1200
900
600
300
0
ASP GLU SER ASN GLY HIS THR ALA ARG
Amino Acid
Stg 1
Stg 4
Stg 8
Stg 9.3
Stg 9.10
IPST
Confidential Information
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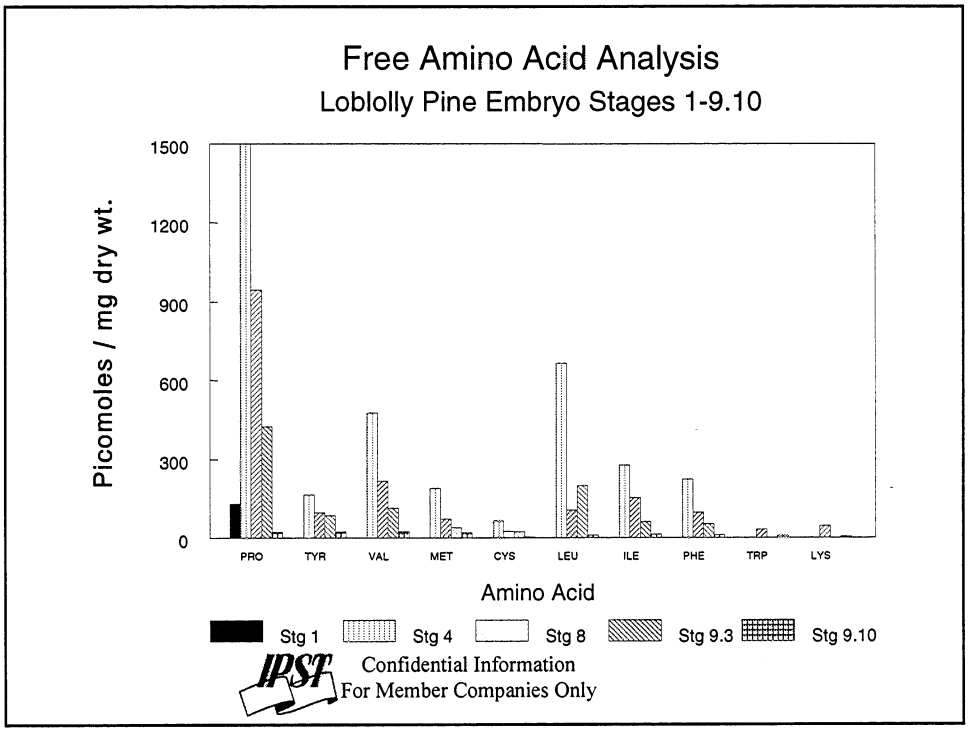
Free Amino Acid Analysis
Loblolly Pine Embryo Stages 1-9.10
Picomoles / mg dry wt.
1500
1200
900
600
300
0
PRO
TYR
VAL
MET
CYS
LEU
ILE
PHE
TRP
LYS
Amino Acid
Stg 1
Stg 4
Stg 8
Stg 9.3
Stg 9.10
IPST
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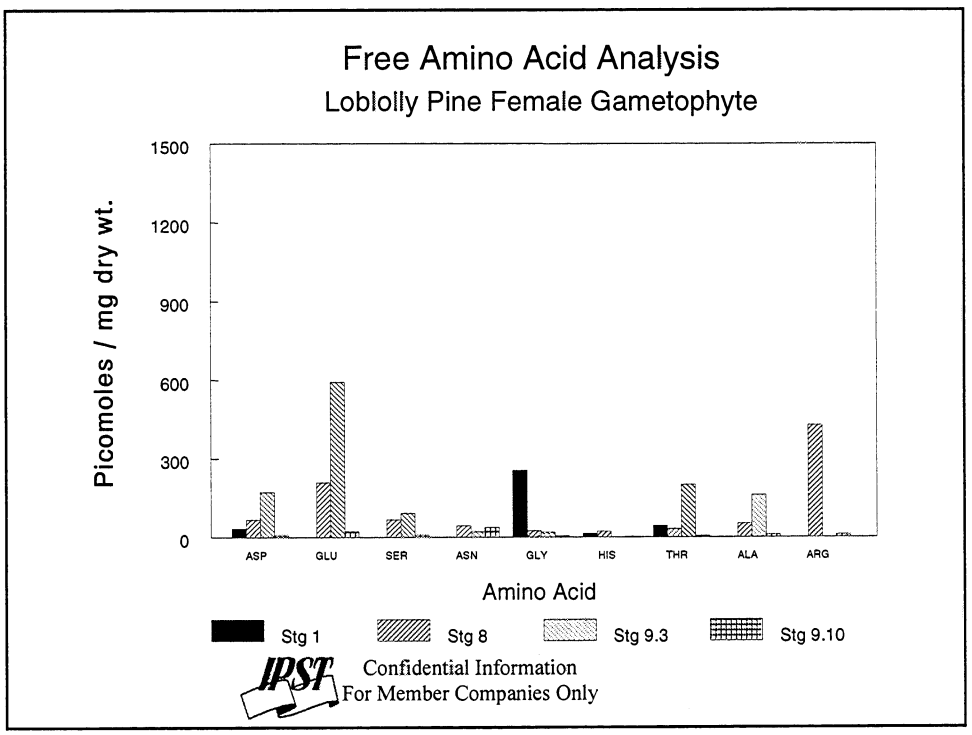
Free Amino Acid Analysis
Loblolly Pine Female Gametophyte
Picomoles / mg dry wt.
1500
1200
900
600
300
0
ASP
GLU
SER
ASN
GLY
HIS
THR
ALA
ARG
Amino Acid
Stg 1
Stg 8
Stg 9.3
Stg 9.10
IPST
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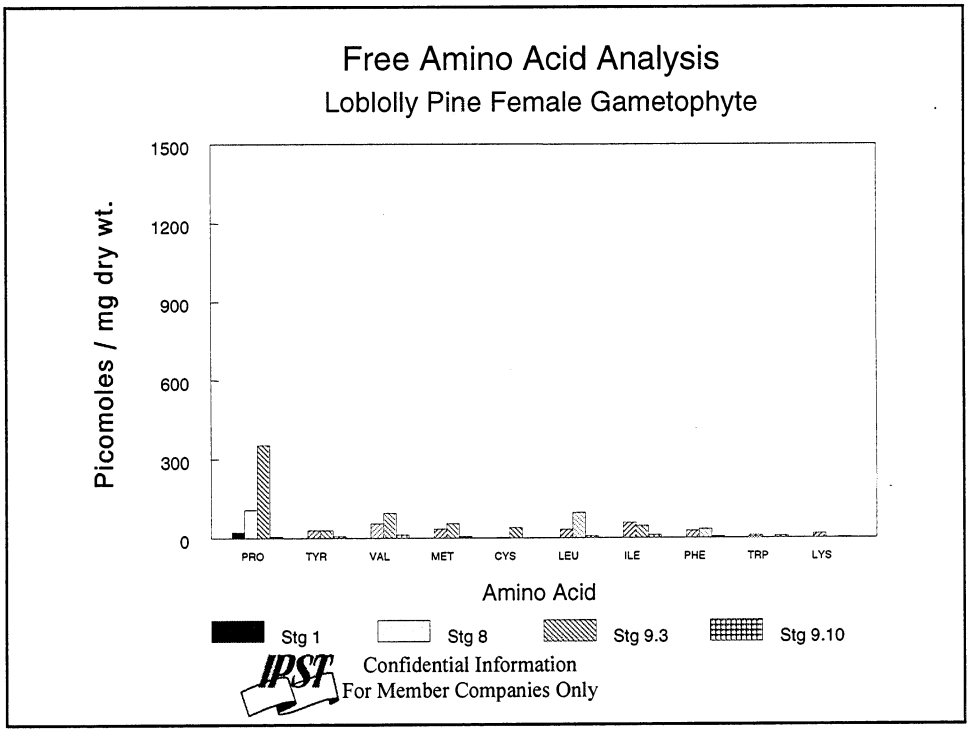


CONCLUSION

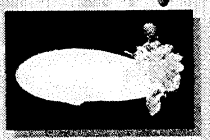
- A free amino acids analysis method has been developed for embryo and female gametophyte tissues of loblolly pine. The instrumentation has been set and is operating well.
- Stage 4and 8 embryos show much greater amounts of free amino acids compared to all other stages. Especially stage 4.
- Free amino acids show increasing and decreasing patterns which are well coordinated with the embryo growth cycle.

Functional Analysis of *Pinus taeda* Zygotic Embryo Germination: *The Effect of Partial Drying and The Acquisition of Desiccation Tolerance*

Gary Peter
Teresa Vales
Sarah Sward
John Cairney
Nanfei Xu
Gerald Pullman

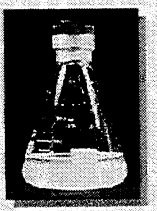
IPST

Somatic Embryogenesis

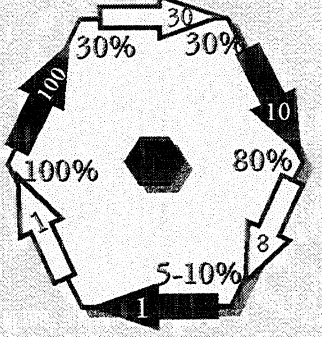


Initiation 15-30%

Conversion 80-100%



Multiplication 100%



Germination 0-50%

Maturation 5-90%

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OBJECTIVES

- Characterize germination rates of immature zygotic embryos with and without partial drying treatments.
- Determine when during zygotic embryo development tolerance to partial drying treatments is acquired.
- Correlate changes in the expression of genes related to desiccation tolerance and acquisition of tolerance to partial drying treatments in both zygotic and somatic embryos.

Materials & Methods

- Genotypes - S4PT6
- Media - Germination 357
- Standard germination protocol
  - 1 week dark then continuous light
- Molecular Analyses
  - Genotypes
  - Methods described previously Xu, Ciarney, Pullman Spring 1998 Pac Report

Experimental Procedures & Design

- Procedures
  - Immature zygotic embryos were dissected from sterile ovules and placed either directly or partially dried for 17 days and then placed onto germination plates.
  - Partial drying treatments were performed in multiwell plate as described in Roberts, *et. al.*, (1990) *Can. J. Bot.* 68 1086-1090.
- Design
  - Germination of stages 7-9.2 was characterized
- Scoring
  - Germinated embryos contained both a root and a shoot
  - After 19 days in light
  - Embryos survived partial drying treatment if cotyledons greened

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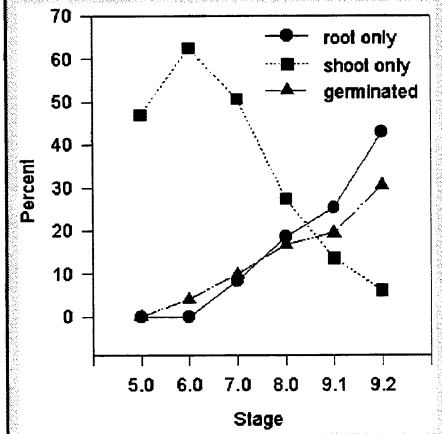
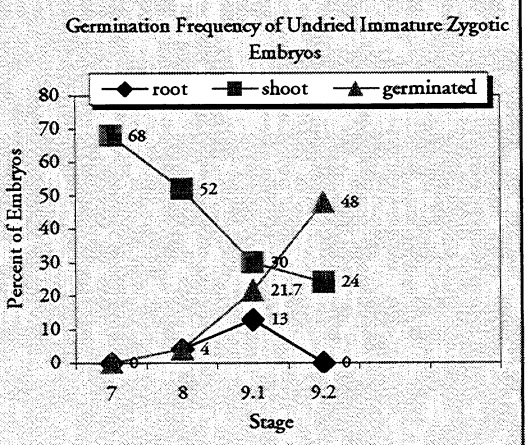
Germination of Undried Immature Zygotic Embryos

UC5-1036 - '97



S4PT6 - '98



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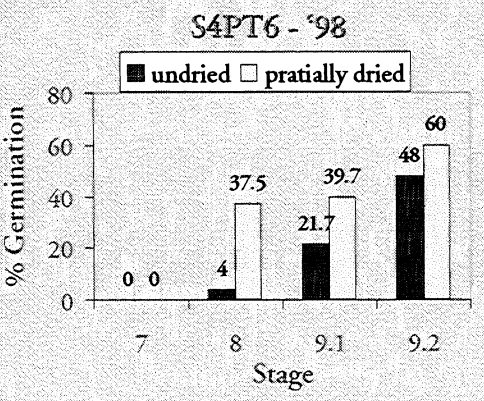
Germination of Undried Embryos

- Root emergence limits germination
- Drying not essential for germination of immature zygotic embryos
- Germination increases with development

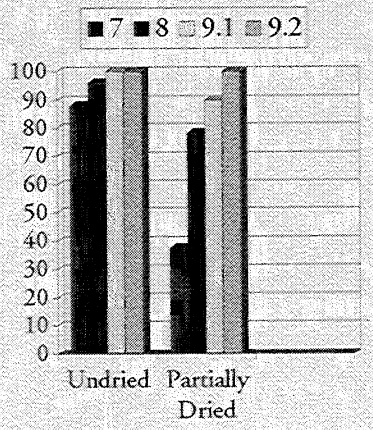
Germination of Partially Dried Immature Zygotic Embryos

- Partial drying was for 2 weeks in the dark
  - Scoring after 21 days in the light
- The more immature the embryo, the larger the % increase in germination after partial drying



Survival of Partial Drying Treatment

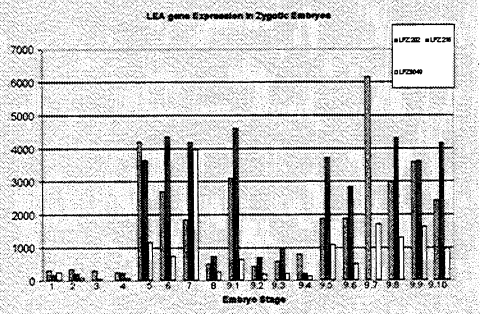
- Most of stage 7 zygotic embryos were not tolerant to partial drying treatment
- Partial desiccation tolerance is acquired by stage 8
- Loss in fresh weight with this method is 25-75%

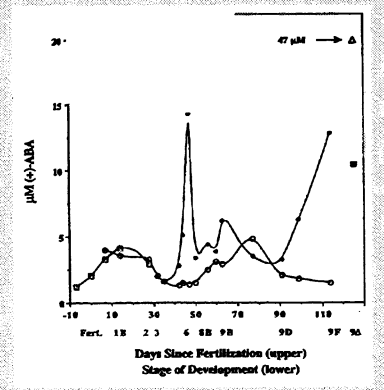


Late Embryogenesis Abundant Genes are Related to Desiccation Tolerance

- LEA genes are ABA regulated and expressed late during embryogenesis
- LEA genes code for proteins that may promote desiccation tolerance
- 3 cDNAs with significant similarity to LEA genes were isolated from *P. taeda*

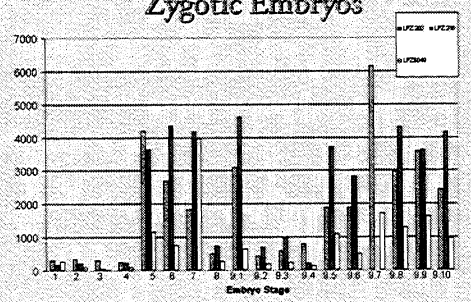
LEA Gene Expression Correlates with High ABA Levels



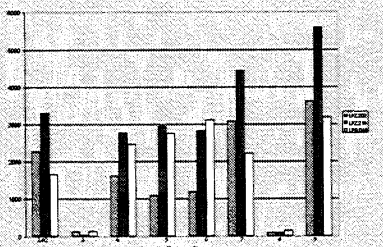


Expression of LEA Genes in Zygotic and Somatic Embryos

Zygotic Embryos



Somatic Embryos

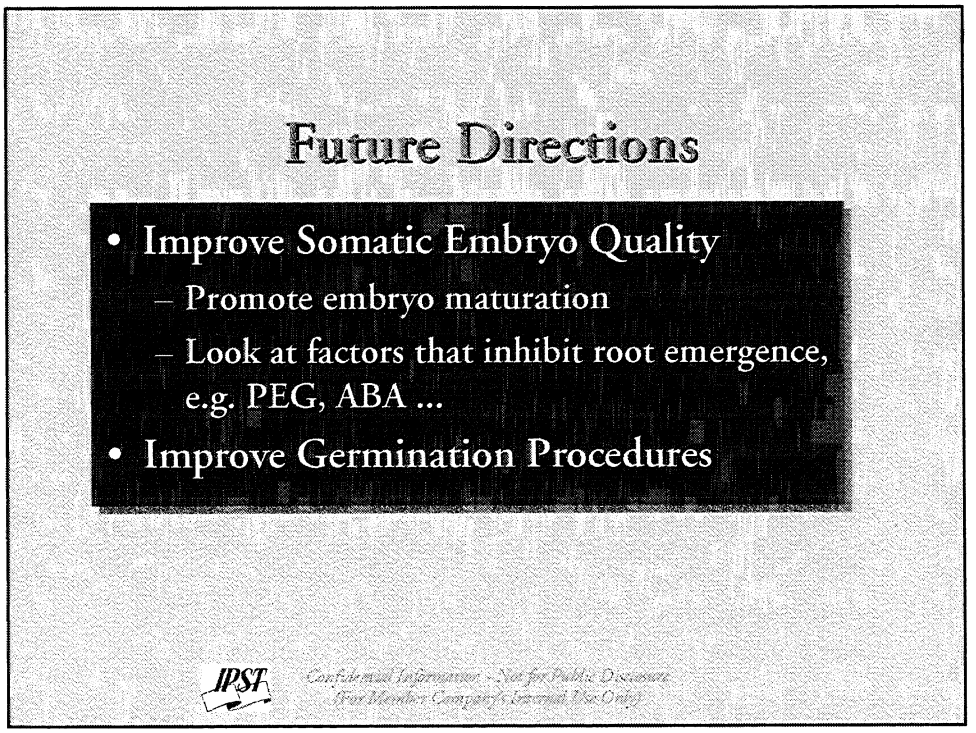


Conclusions

- Germination potential of zygotic embryos increases with maturity whether embryos are partially dried or not.
- Partial drying promotes immature embryo germination once some level of desiccation tolerance is obtained
- Desiccation tolerance acquired by stage 8

Conclusions

- LEA gene expression occurs prior to the acquisition of desiccation tolerance and is probably ABA regulated in *Pinus taeda.*
- Somatic embryos acquire desiccation tolerance, but other factors which are not altered by partial drying treatments limits germination potential


Future Directions
• Improve Somatic Embryo Quality
– Promote embryo maturation
– Look at factors that inhibit root emergence, e.g. PEG, ABA ...
• Improve Germination Procedures
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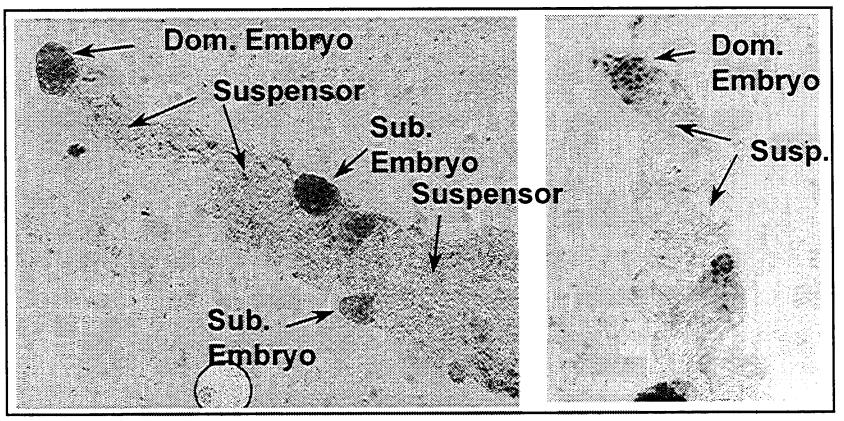
Role of the Suspensor in Early Embryo Development

Gene isolation and expression

John MacKay, Heidi Schindler,
Christina Perfetti, John Cairney, Gerald Pullman

Background: Why the suspensor

The Suspensor of Immature Zygotic Embryos



Goal and Approach

- Goal: **Increase understanding of function and role of the suspensor in zygotic and somatic embryos with the aim of improving somatic embryogenic cultures**
- Objectives:
  - Identify genes that are more abundant in the suspensor or are specific to the suspensor
  - Obtain cDNAs clones that are differentially expressed in the suspensor
  - Verify tissue specificity of cDNAs

Previous Results: Isolation of Suspensor cDNAs

Embryo Dissection

- Construction of cDNA libraries enriched for suspensor genes (stage 3 and stage 4)
- Screened and characterized cDNA Clones & Confirmation of specificity
  - Dot Blot Analysis of 240 clones
- DNA sequence analysis identified several storage protein genes that were very abundant in the suspensor libraries.

Isolation of Suspensor cDNAs - Progress

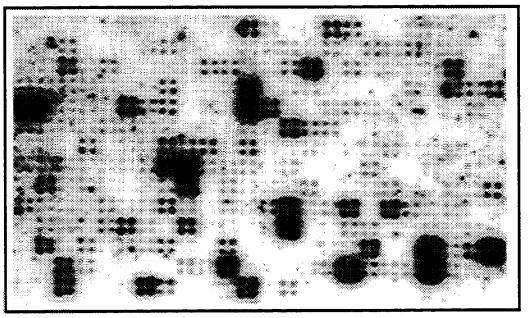
1. Further screening of 341 cDNA clones
2. Sequencing and database searching of suspensor abundant and low abundance cDNAs
3. Estimation of relative transcript (mRNA) abundance of major storage proteins
4. Screening of differentially expressed embryo cDNAs (Xu et al., Spring PAC 1998)

Suspensor Abundant Genes

Gene	Organism	Probability Score	Transcript Abundance in the *Suspensor* Stage 3	Stage 4
•Albumin 1 (seed storage protein)	White pine	10^{-86}	++	++
•Albumin 3 (seed storage protein)	White pine	10^{-112}	++,+++	+
•Albumin 4 (seed storage protein)	White pine	10^{-110}	++,+++	+
Alpha tubulin	Barley	10^{-61}	+, ++	
•Globulin (seed storage protein)	White pine	10^{-35}	+, ++	
•Uncharacterized embryo protein	Black spruce	10^{-66}	+	+++
•Legumin (seed storage protein)	Douglas fir	10^{-90}	++,+++	
•Vicilin (seed storage protein)	White spruce	10^{-138}	+++	
•Pine embryogenesis protein PRE87	Radiata pine	10^{-14}	+,+++	+
Cysteine proteinase inhibitor(defense)	Soybean	10^{-17}	++	
Hin 1 gene (defense related)	Tobacco	10^{-10}	++	
Putative protein	*Arabidopsis*	10^{-11}	+	+++
Cysteine protease (stress related)	Douglas fir	10^{-149}	+	
Late embryogenesis abundant protein	White spruce	10^{-102}	+	+++
Catalase isoenzyme 2	Upland cotton	10^{-94}	+	++
Catalase 1	*Arabidopsis*	10^{-52}	+	++
Clone 404	No hits produced		+	
Clone 406	No hits produced		+	
Clone 514	No hits produced		+,++	
Clone 539	No hits produced		+,+++	

Relative Abundance of Embryo cDNAs in the Suspensor and Megagametophyte

- Selected cDNA clones
  - 1. From subtractive hybridizations
  - 2. Differentially expressed embryo cDNA clones (Xu et al. PAC spring 1998).
- Probed with cDNA populations
  - Suspensor, Megagame-tophyte and Embryo "head".
  - Developmental stage 3 or 4.



Gene arrays of Xu et al. 1998

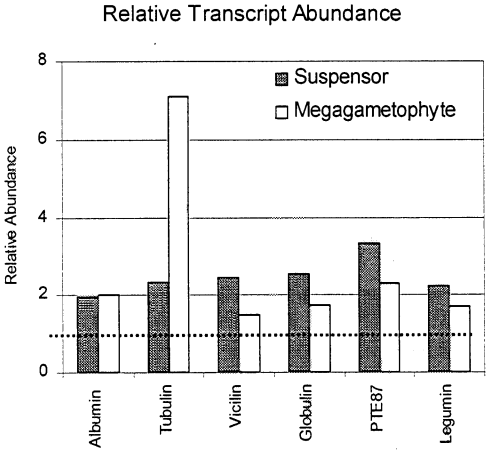
Exposed for 72 hours

Relative Abundance of Embryo cDNAs

ESTIMATION OF RELATIVE ABUNDANCE

- Hybridization signal intensity measured from X-Ray film image:
  - scanned image of film
  - image analysis software
- Normalization with a ribosomal RNA (rRNA) clone
  - Normalized Intensity of clone X = $\frac{\text{Intensity of clone X}}{\text{Intensity of rRNA}}$
- Transcript (mRNA) abundance in Suspensor and Megagametophyte is determined relative to the E. head
  - Relative abundance = $\frac{\text{Normalized Susp. or Mega. Intensity}}{\text{Normalized Head Intenstiy}}$

Relative Abundance of Embryo cDNAs



- Suspensor and Megagametophyte follow the same trend for transcript abundance these genes
- Expression of storage proteins is primarily controlled at the level of transcription

Relative Abundance of Embryo cDNAs*

MORE ABUNDANT

Gene	Relative Abundance	
	Suspensor	Gametophyte
Albumin 1	1.7	1.8
Elongation Factor-2		2.5
LEA protein (2 cDNAs)	2.2, 2.8	3.0, 4.0
Oxygen evolving complex (2 cDNAs)	+++	
Plasma memb. ATPase		2.0
SnRNA associated prot.	2.0	1.6
Transcript. regulator	2.1	1.2
Ubiquitin-like protein	2.5	
Yeast membrane prot.	2.5	
9 cDNAs, no hits	+	
8 cDNAs, no hits	+	+
3 cDNAs, no hits		+
Ecdysome-inducible protein E75	1.6	0.3

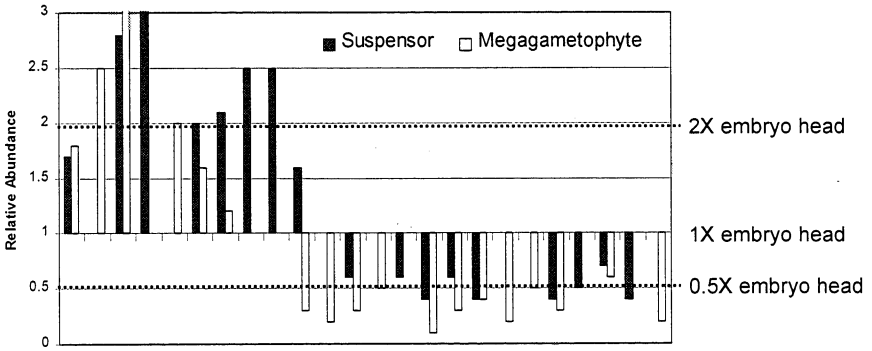
LESS ABUNDANT

Gene	Relative Abundance	
	Suspensor	Gametophyte
Actin		0.2
B2 protein from carrot	0.6	0.3
Dynamin-like GTP-bind.		0.5
EST similar to Insulin	0.6	
GA regulted pro. GASA5	0.4	0.1
Histone 3	0.6	0.3
Malate oxidorecutase		0.4
Mouse embryo ectoderm	0.4	
Protein kinase		0.2
Ribonuleoprotein Sm D3		0.5
Ribosomal protein L23a	0.4	0.3
Serine kinase (human)	0.5	
Trans. Elong. factor 1-a	0.7	0.6
Transmembrane WD 40 type 1 proteing	0.4	
Voltage depend. anion channel		0.2
5 cDNAs no hits	-	
9 cDNAs no hits	-	-
12 cDNAs no hits		-

*cDNAs from Xu et al. 1998

Relative Abundance of Embryo cDNAs*

- Interpretation of relative abundance values:
  - Threshold? Repeatability?
- Value of data obtained from film...



Ongoing experiments

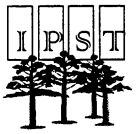
- **Standardization of procedures for array construction, probe preparation and data manipulation. L. Ge and J. Cairney**
- **Verify gene expression results in other genotypes**
- **Analysis of gene expression in somatic embryogenic lines**

Future Directions

- Suspensor proliferation and differentiation in somatic embryogenesis
  - Gene expression
  - Effect of tissue culture conditions on suspensor development
  - Suspensor - Embryo head interactions
  - Pre-proposal to DOE
- Develop markers for suspensor cells
- Develop hypotheses to improve embryo quality

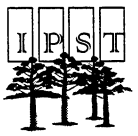
The Suspensor in Somatic Embryogenic Liquid Culture

→ "Suspensor" Cells



Analysis of a Late Stage-specific Clone of Loblolly Pine by a Non-radioactive Method: Developing Detection Tools

Barbara Johns and John Cairney



Methods Objectives: Technique Development

- **Develop an alternative protocol to the conventional methods using radioactive isotopes**
- **Determine sensitivity of method**
  Is it as sensitive as radioactive protocols?
  Can the method detect rare mRNAs or single copy genes?

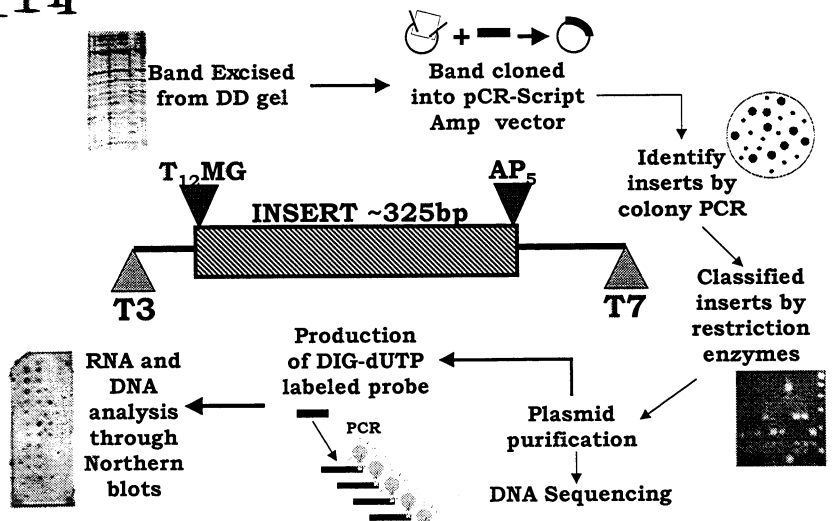
Project Goals: Clone Characterization

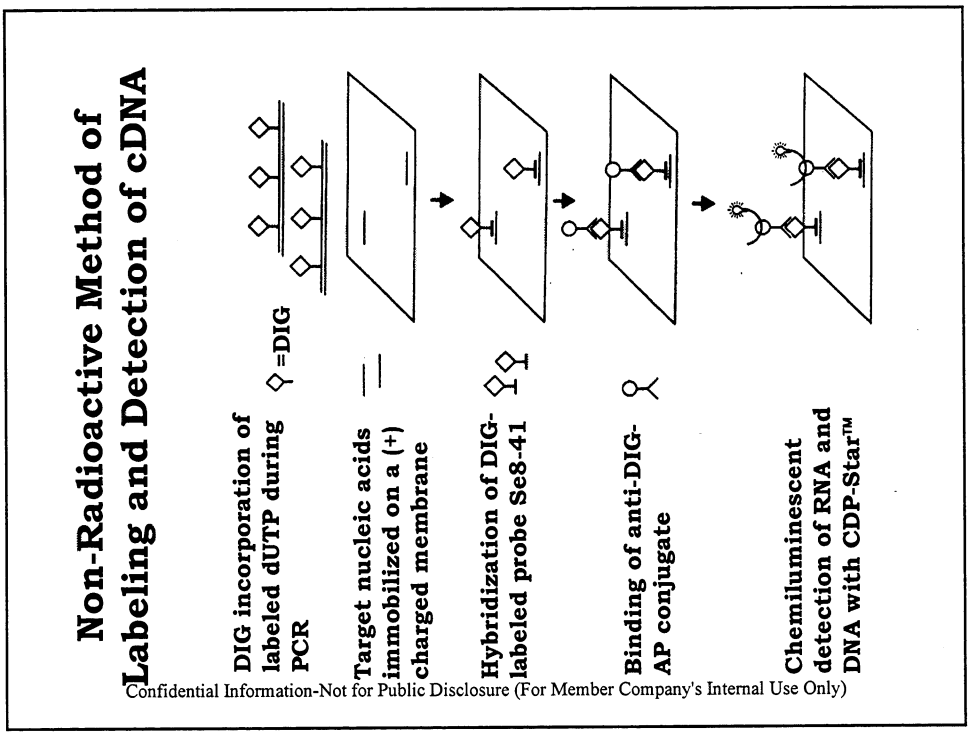
Determine

- size of message
- stage specificity
- quantitative comparisons

  Can the method detect amount of message?

Origin of Stage-specific Probe Se8-41




Non-Radioactive Method of Labeling and Detection of cDNA
DIG incorporation of labeled dUTP during PCR
=DIG
Target nucleic acids immobilized on a (+) charged membrane
Hybridization of DIG-labeled probe Se8-41
Binding of anti-DIG-AP conjugate
Chemiluminescent detection of RNA and DNA with CDP-Star™
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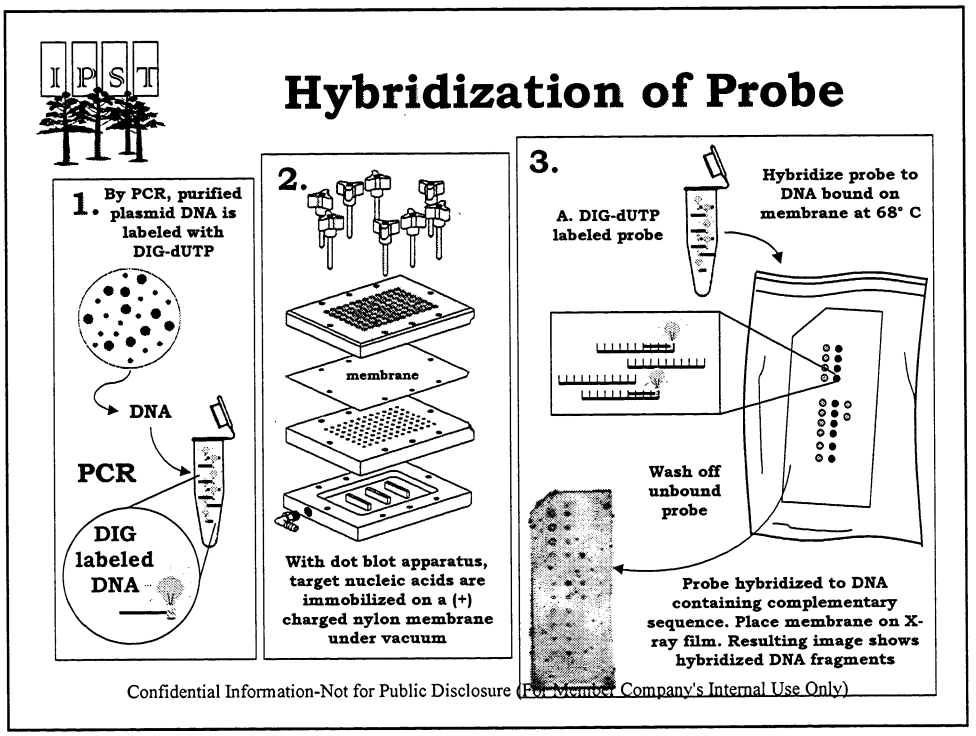
I P S T
Hybridization of Probe
1. By PCR, purified plasmid DNA is labeled with DIG-dUTP
DNA
PCR
DIG labeled DNA
2.
membrane
With dot blot apparatus, target nucleic acids are immobilized on a (+) charged nylon membrane under vacuum
3.
A. DIG-dUTP labeled probe
Hybridize probe to DNA bound on membrane at 68° C
Wash off unbound probe
Probe hybridized to DNA containing complementary sequence. Place membrane on X-ray film. Resulting image shows hybridized DNA fragments
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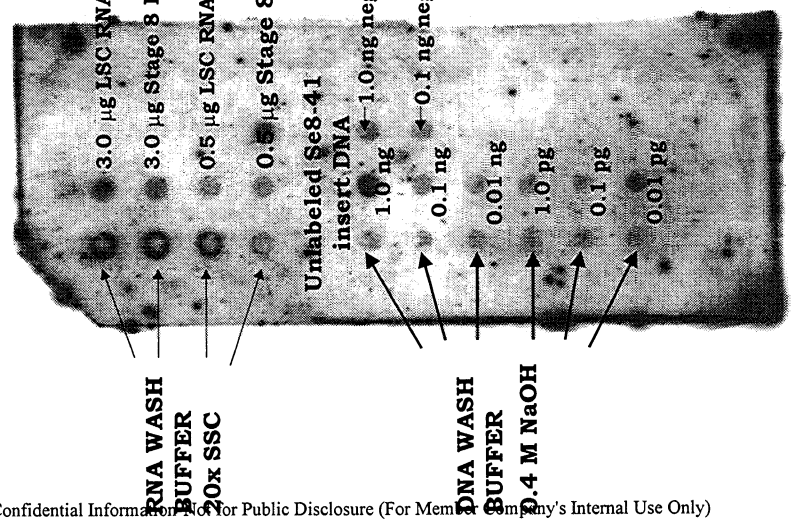
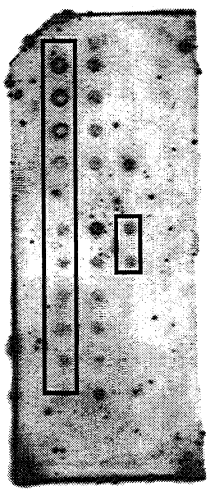
Non-radioactive Dot-Blot Northern Hybridization



Optimization of Protocol

- **Non-specific binding**
  - **-buffer: reduce concentration**
  - **-negative controls**
- **Amplify with primers $T_{12}MG$ and AP_5**



Future Work

- Further refinement of protocol
- Determine stage specificity of clone
- Determine size of message
- Quantify message

Future Work

- Chemiluminecsent vs. radioactive protocol
- Camera vs. film
  - shorter exposure time
  - quantify amount of message

EXTERNALLY FUNDED RESEARCH in 1998-1999 SUPPORTING F010

John Cairney
Gerald Pullman
Gary Peter
Lin Ge
Vincent Ciavatta

March 25-26, 1999

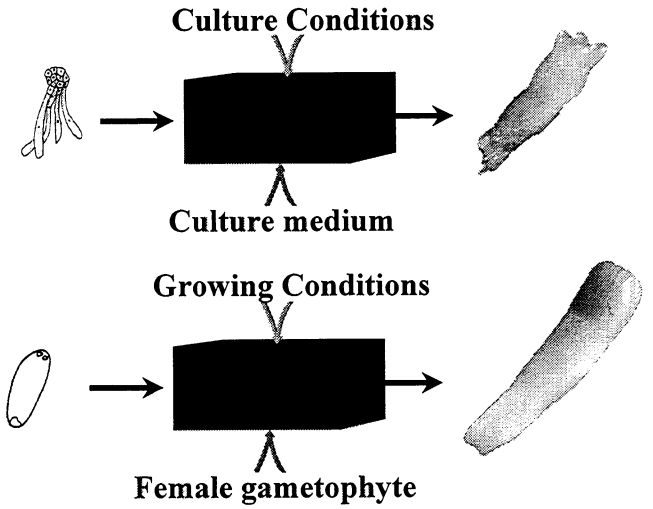
IPST

Monitoring Gene Expression During Loblolly Pine Embryogenesis

John Cairney

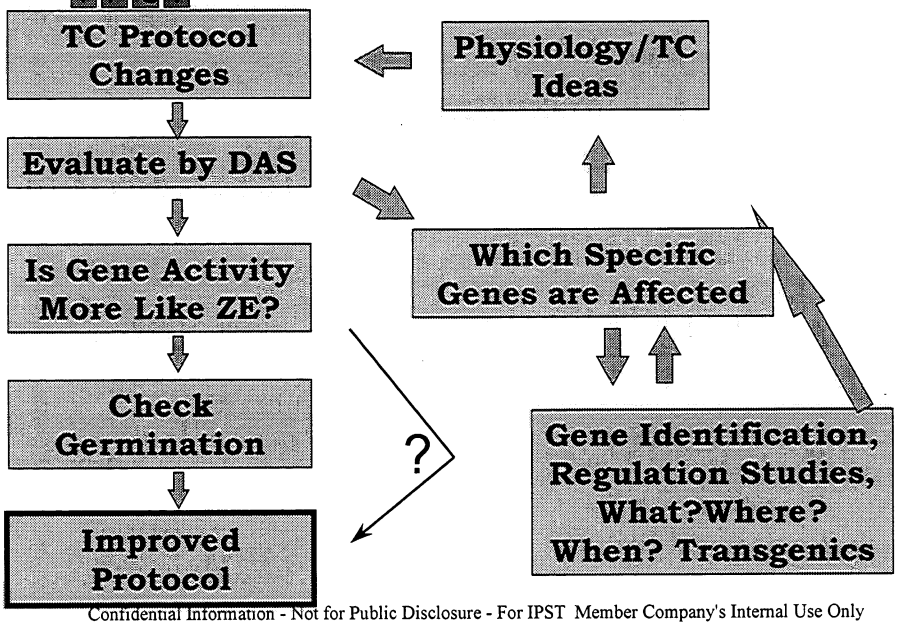
IPST

The Difference between SE and ZE Lies in Gene Expression



How Gene Expression Studies Will Help SE Protocol Development

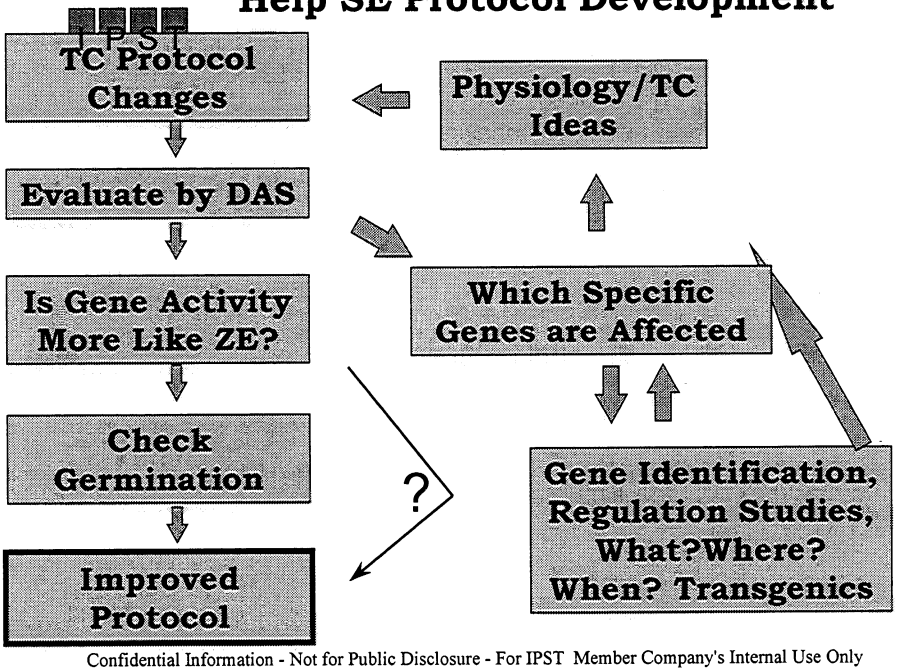
IPST



IPST Monitoring mRNA for Large Numbers Genes by DNA Array

- We have isolated over 450 clones of genes whose activity changes as embryos develop
- All are potentially informative
- Differential Display lets you view a subset of these genes (plus many of less interest), repetitions needed for a complete picture
- DNA Arrays focus upon your genes of interested

How Gene Expression Studies Will Help SE Protocol Development



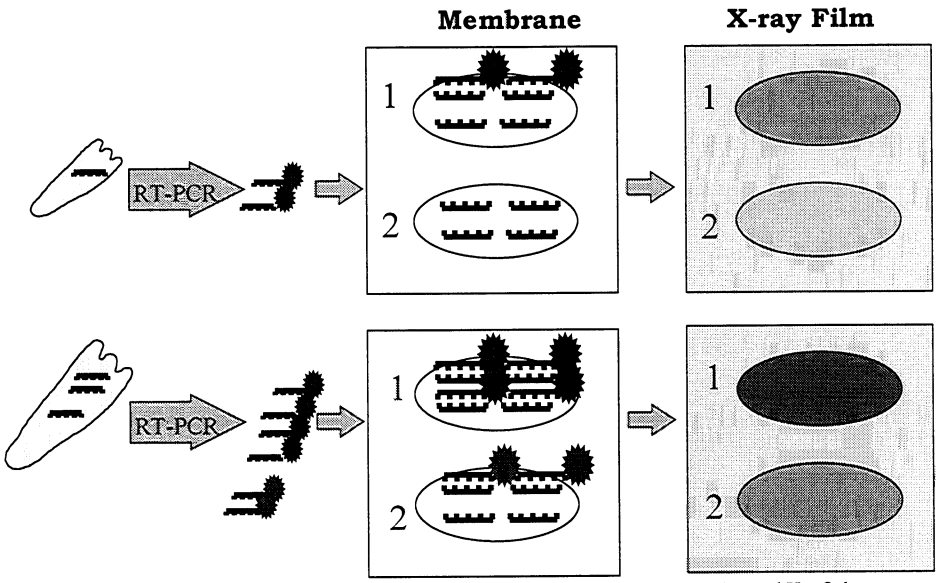
IPST Monitoring mRNA for Large Numbers Genes by DNA Array

- We have isolated over 450 clones of genes whose activity changes as embryos develop
- All are potentially informative
- Differential Display lets you view a subset of these genes (plus many of less interest), repetitions needed for a complete picture
- DNA Arrays focus upon your genes of interested

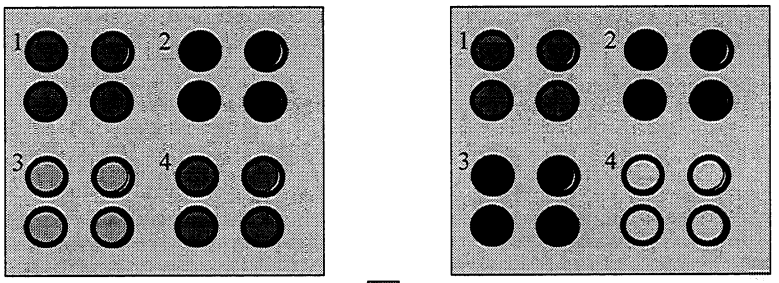
Establishing a Database of mRNA Levels During Embryogenesis

- By determining quantitatively the mRNA levels for hudreds of genes we get insight into the natural process (zygotic)
- We can compare this to the somatic process
- We can develop new ideas
- We can compare new protocols to see how closely the somatic embryos resemble the zygotic embryos

Principle of Dot Array Southerns: Summary



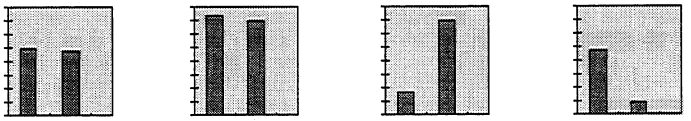
Quantitation and Graphical Display Quickly Reveal Patterns



Stage 6 Probe

Stage 9 Probe

Quantitate and Graph



Clone 1 Clone 2 Clone 3 Clone 4

Problems With Use of X-ray Film

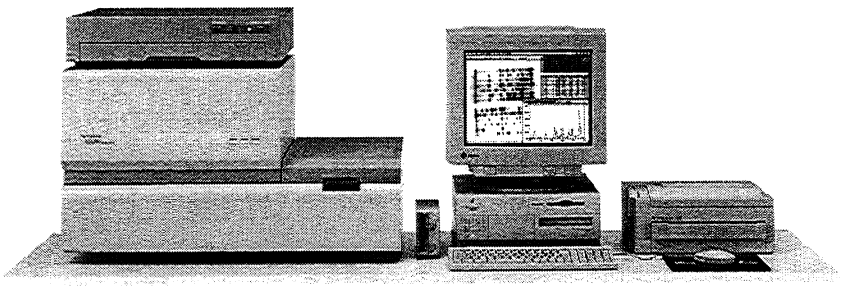
- **Detection is only linear over a certain range**
- **Speed: Large differences in signals intensity are reliably detected but for less marked differences different exposures were needed for comparisons**
- **Resolution: Certain signals 'bled' into the field of neighboring signals**

We Have Purchased an Imaging Detection System (BAS 1800, Fuji) to Resolve Problems

- **Detection: Linear over entire range and 5 orders of magnitude**
- **Speed: BAS 1800 Is Rapid and Sensitive (10-20X faster than film)**
- **Resolution: 50/100/200 pixels**

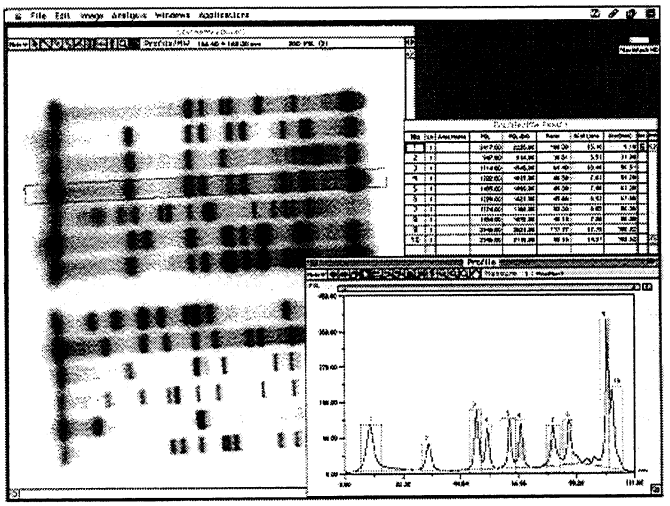
Phosphoimager

BAS-1800

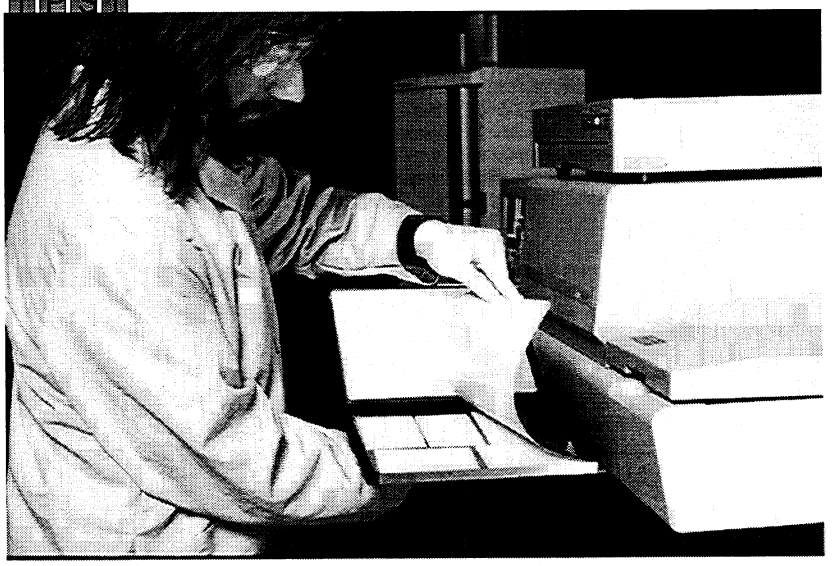


FUJIFILM
I&I - Imaging & Information
See your data in a whole new light.

Analysis By BAS 1800 Phosphoimager



Southern Blotting



IPST

Non-Radioactive Detection Systems

- **The Safety Issues and Cost of Disposal associated with Radioisotopes cause us to consider alternatives**
- **CCD Cameras can detect Chemiluninescent and Fluorescent Signals**
- **Range is Linear for 4 order of magnitudes**
- **Camera could be mounted on top of microscope in future, when microscope slide-formatting of DNA arrays becomes cheaper**

IPST

LAS 1000 Detection System

Now see faint-light images with high sensitivity and high resolution.

The LAS-1000 Single System captures chemi-luminescent, fluorescent and chemifluorescent images.

When a fluorescent or chemifluorescent image is captured with this system, a blue-LED epi-illuminator can be used to excite the related dyes. This optional epi-illuminator operates at a wavelength of 470nm. The Fujifilm LAS-1000 accepts Membrane or Gel samples up to 25x25cm size and allows use of a variety of reagents:

- Chemiluminescence – Use CDP-Star® ECL™
- Fluorescence – Use EtBr, SYBR® Green I & II, FITC
- Chemifluorescence – Use AttoPhos™

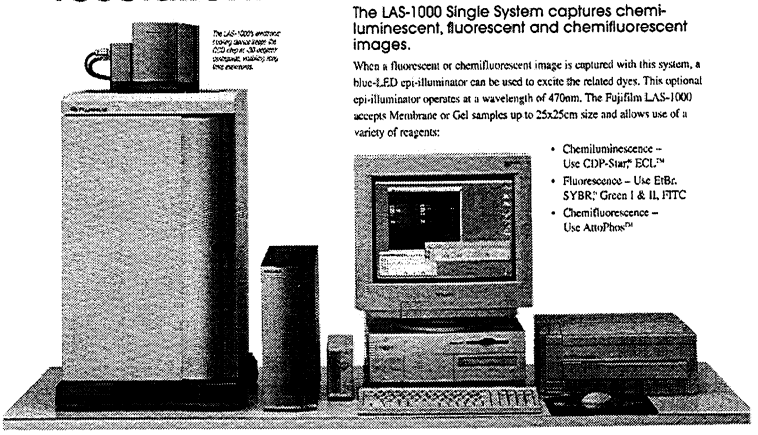


Diagram of LAS 1000 Detection System

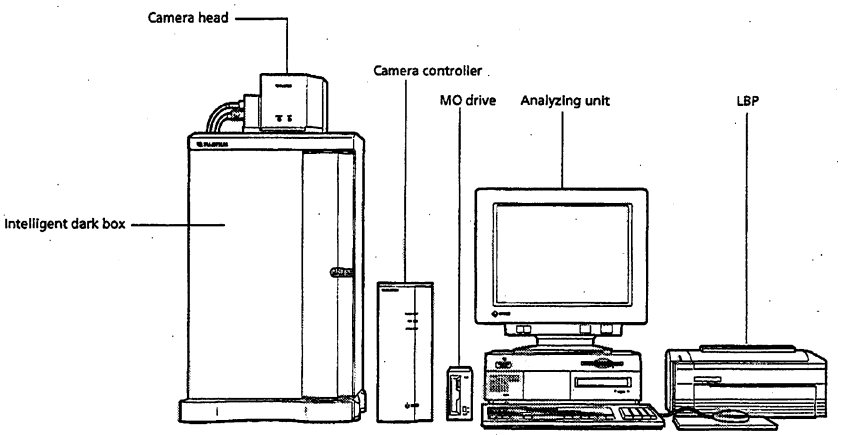


Fig. 4.1 Hardware Configuration (Example: Macintosh version)

Camera Position in LAS 1000

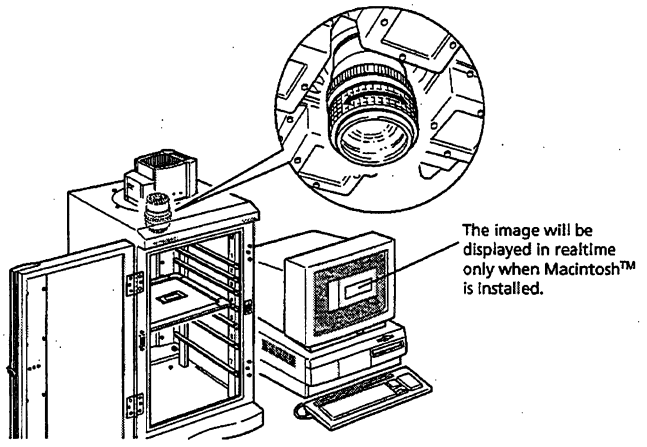
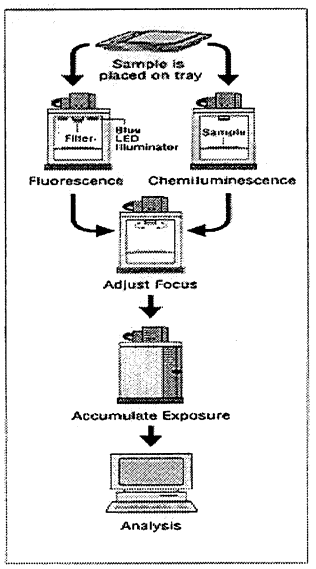


Fig. 5.21 Focusing

Process of Detection With LAS 1000

LAS-1000 analysis process



Additional Illumators Permit Fluoresce to be Detected

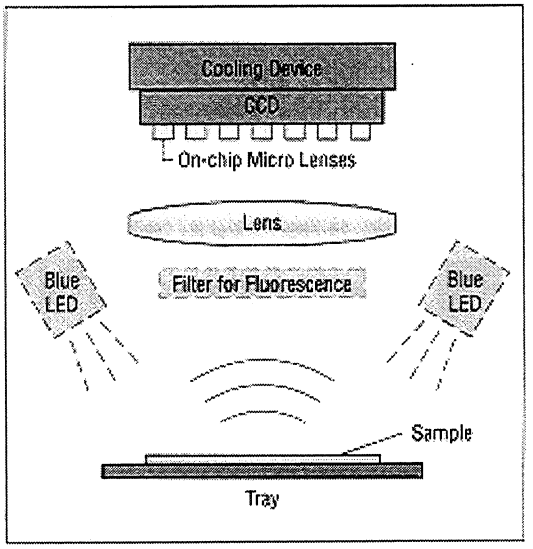
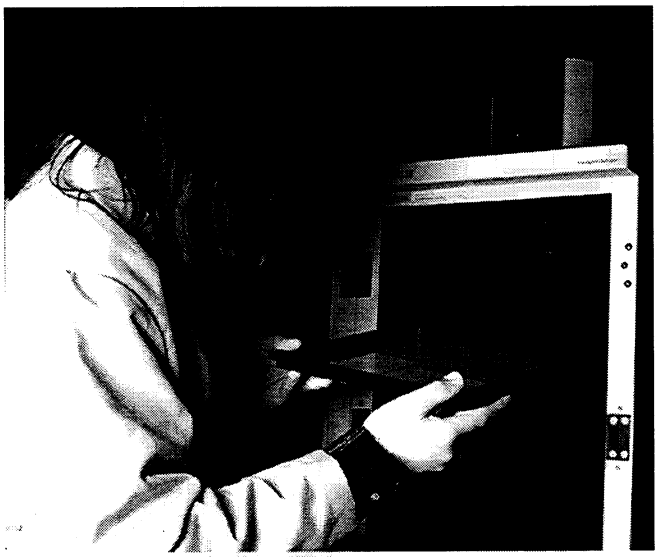


Image capture is possible through both chemi-fluorescence and fluorescence.

IPST

Placing Filters in LAS1000



IPST

Approaches With Gene Regulation Studies

Vincent Ciavatta

Expression and Function of Gene in model Transgenic Plants

Vincent Ciavatta

Gene Function Studies, in situ hybridizations, function of protein in vivo

Cloned and Sequenced cDNAs

Stage Specific Genes, Specific Primers

Bobbie Johns

Early Development Stage Specific Markers

Vincent Ciavatta, Bobbie Johns

Comparison to Embryogenesis in other Plants

Dot Array Southerns

Lin Ge

Improving Somatic Embryogenesis in Loblolly Pine by cDNA Micro-array Techniques

Lin Ge
Nanfei Xu
Gerald Pullman
John Cairney

Forest Biology
Institute of Paper Science and Technology
500 10th Street NW, Atlanta, GA 30318

Sponsored by IPST Member Companies and GA Consortium

Program Goal:

To develop methods for producing loblolly pine Somatic Embryos which , in quality, resemble Zygotic Embryos

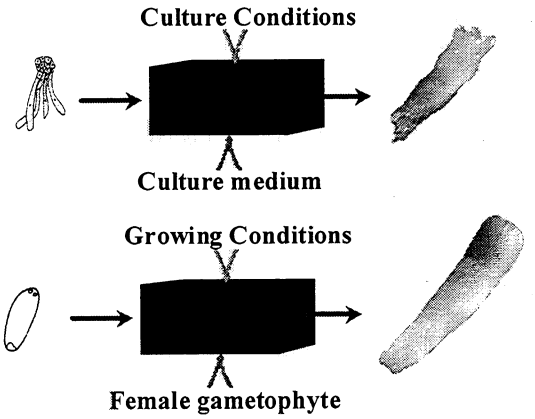
Problems:

Several aspects of Loblolly pine Somatic Embryos are suboptimal Maturation, Germinationand Maintenance in Liquid Culture (culture decline) are among these.

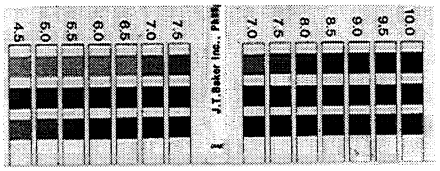
Solutions:

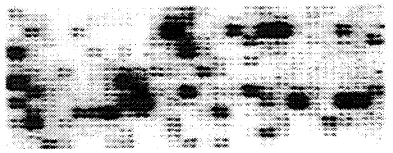
Using Gene Expression Information as New Tools to develop the New solutions to these problems

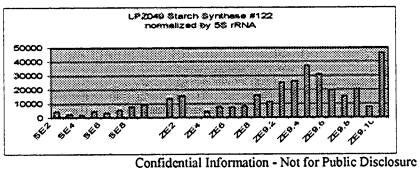
The Difference between SE and ZE Lies in Gene Expression

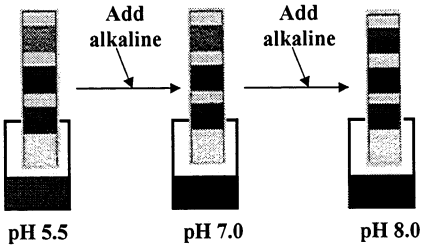


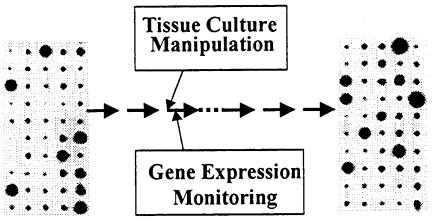
We Desire a Continuos-Monitoring Process Which Is As Quick and Simple to Use as pH Paper





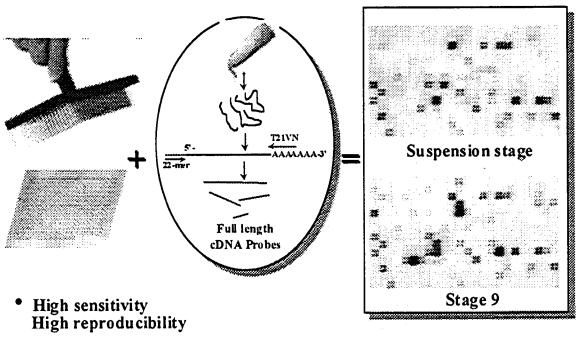




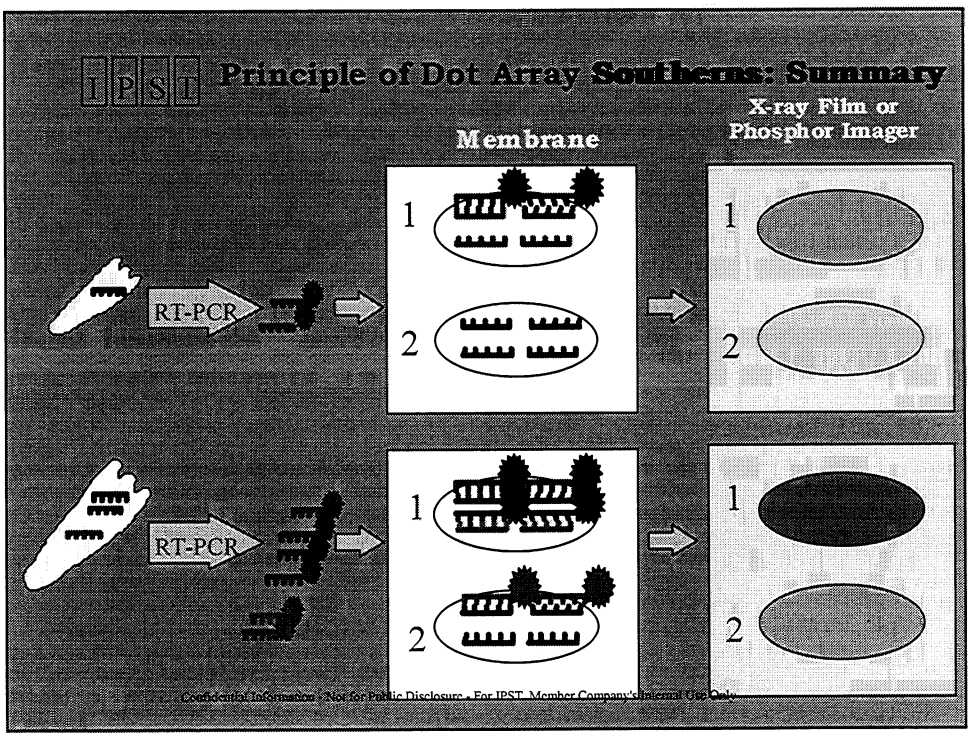


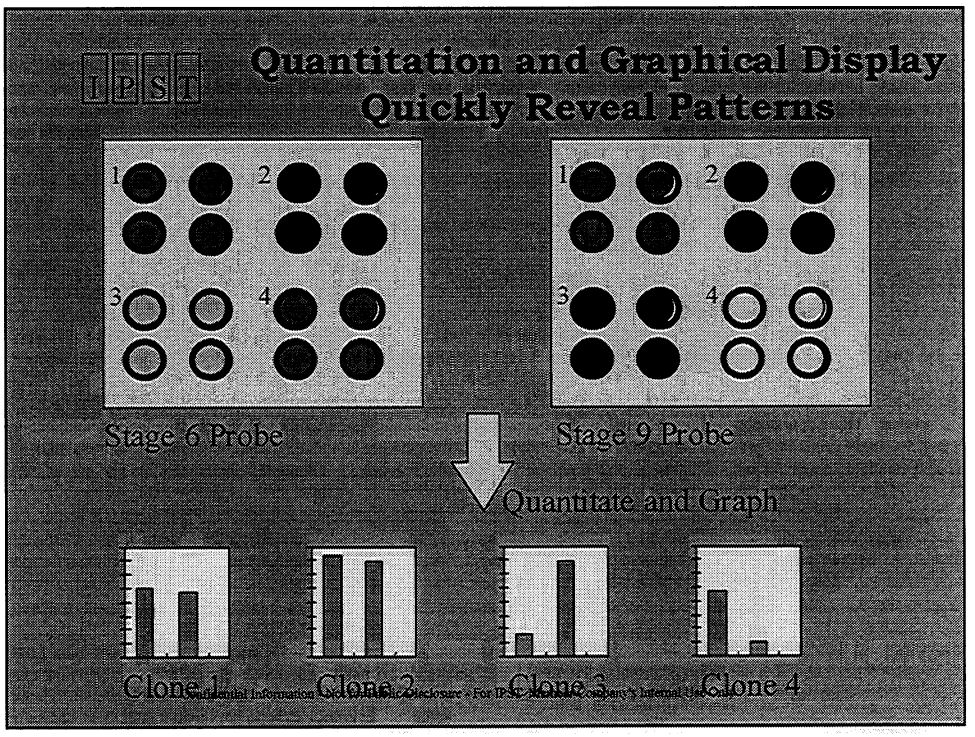
cDNA Micro-Array Technique is a Powerful Tool for Monitoring the Expression of Large Numbers of Genes at Simultaneously

Micro-Array Southern with Amplified Full Length cDNA



- High sensitivity
  High reproducibility

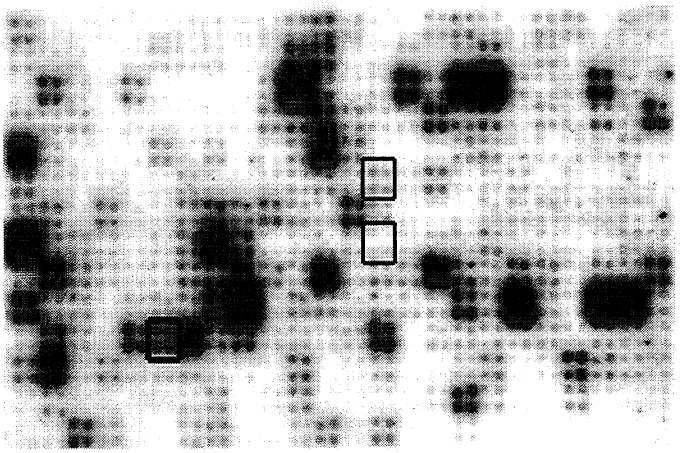




Preliminary Results Are Encouraging But Quantitative Methods Need Improvement

- Large differences in signals intensity are reliably detected but for less marked differences quantitative methods are inconsistent
- Differences in Dot Placement and Probe Synthesis may be responsible
- Method Refinement is our next big goal
- Signals on X-ray film become saturated
- Detection is linear over a limited range
- Software for this system has limited usefulness

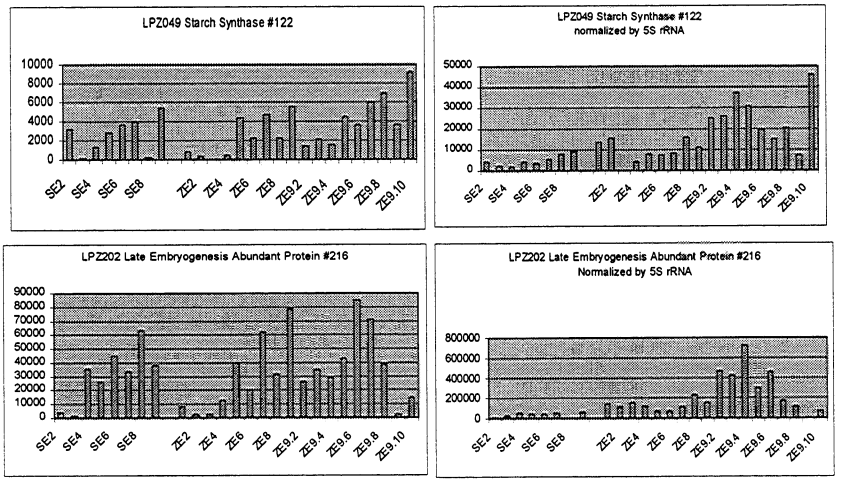
Example of quantify the intensity of cDNA micro-array signals by x-Ray film and Gel-Pro software



Clone LPZ065 5S ribosomal rDNA as control;
Clone LPZ049 starch synthase;
Clone LPZ202 Lae abundant protein

Quantification of cDNA micro-arrays: Normalization Clarifies Trends in Expression

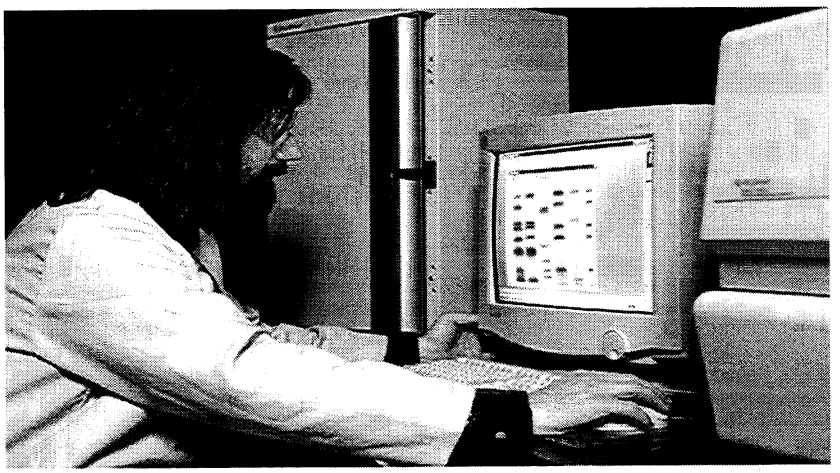
(Signals by x-Ray film and Gel-Pro software)



Without normalization With normalization

To solve the problem:

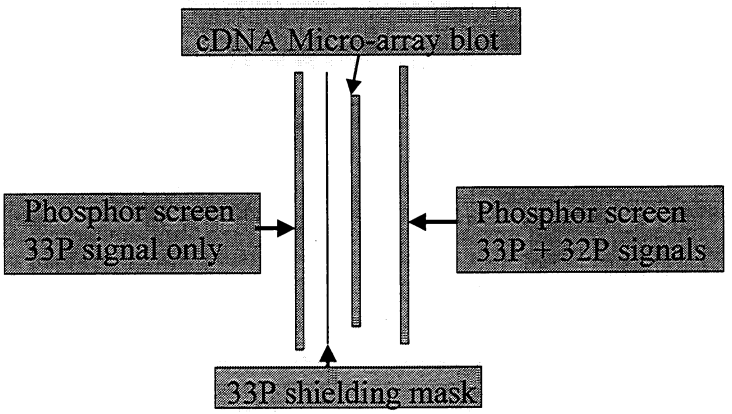
- **Constituitively expressed genes need to be incorporated in the cDNA Micro-arrys as controls to eliminate the experimental error.**
- **Experiments need to be repeated multiple times to obtain credible data.**
- **And .....**



•Phosphor Imager is used to accurately quantify the gene expression levels

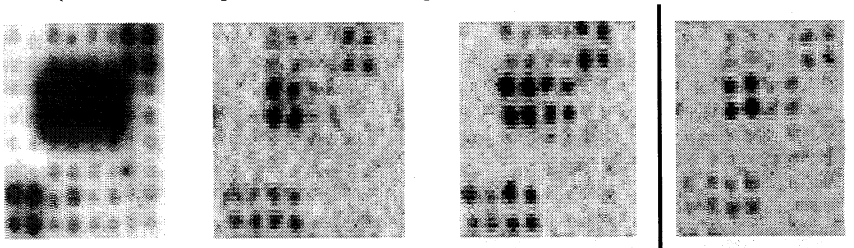
Double Labeling

Simultaneous detection the gene expression in two-culture conditions or two developmental stages could be done by using 32P and 33P labeled probes in the same experiment

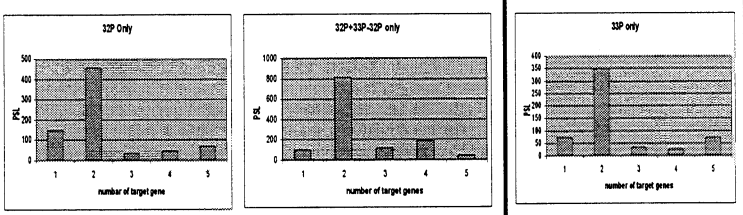


Preliminary Results Using Phosphor Imager and 33P , 32P Labeled BC-1 ZE 9.10 probes

(each value is represented the average of two individual experiments)

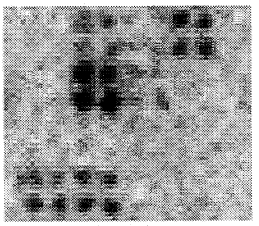


X-ray film (from N.Xu) | 32P | 33P + 32P | 33P only

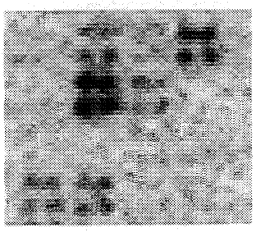


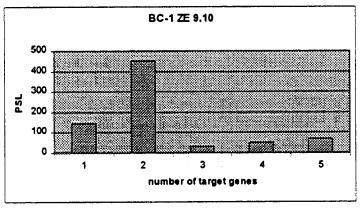
Preliminary Results Using Phosphor Imager Using 32P labeled BC-1 ZE 9.10 cDNA and UC-5 ZE 9.9 cDNA as Probes (each value is represented the average of two individual experiments)

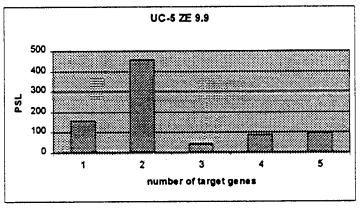
BC-1 ZE



UC-5 ZE





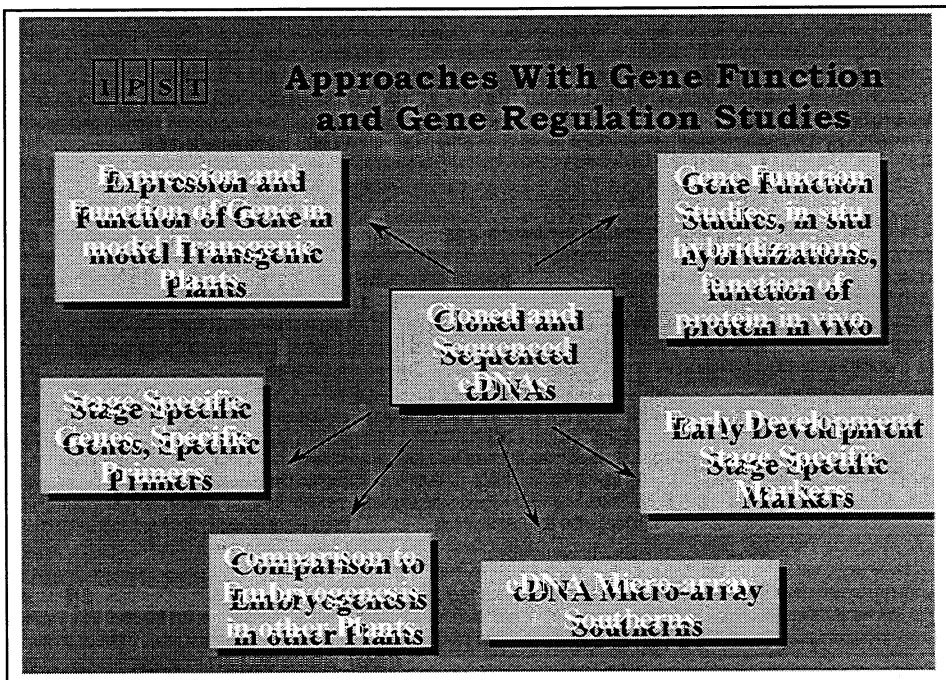


Milestones of the Project

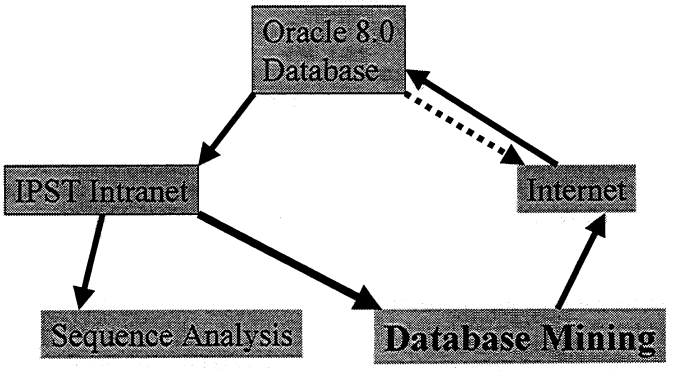
- ✓ **Develop a highly sensitive method of RNA differential display that accommodates minute amount of materials**
- ✓ **Clone a few hundred of cDNAs that are putatively embryo specific.**
- ✓ **Develop a method that can easily and reliably detect the expression of cloned cDNAs in the embryos**
- **Obtain the expression patterns of the cloned cDNAs in the zygotic embryos at different stages**
- **Monitor and manipulate gene expressions in somatic embryos**
- **Study the function and regulation of some of the genes**

Future Goals

- Expanding DNA arrays to include cDNAs recently cloned inour laboratory as well as cDNAs for additional important regulatory genes obtained from public Genebanks
- Establishing a Database of the transcript levels of several hundred genes during loblolly pine embryogenesis



New Approaches With Gene Function and Gene Regulation Studies



Loblolly Pine Embryogenesis: cDNA Cloning, Expression Analysis, and Promoter Cloning of Early Embryo Abundant mRNAs

Vincent Ciavatta
Gerald S. Pullman
John Cairney

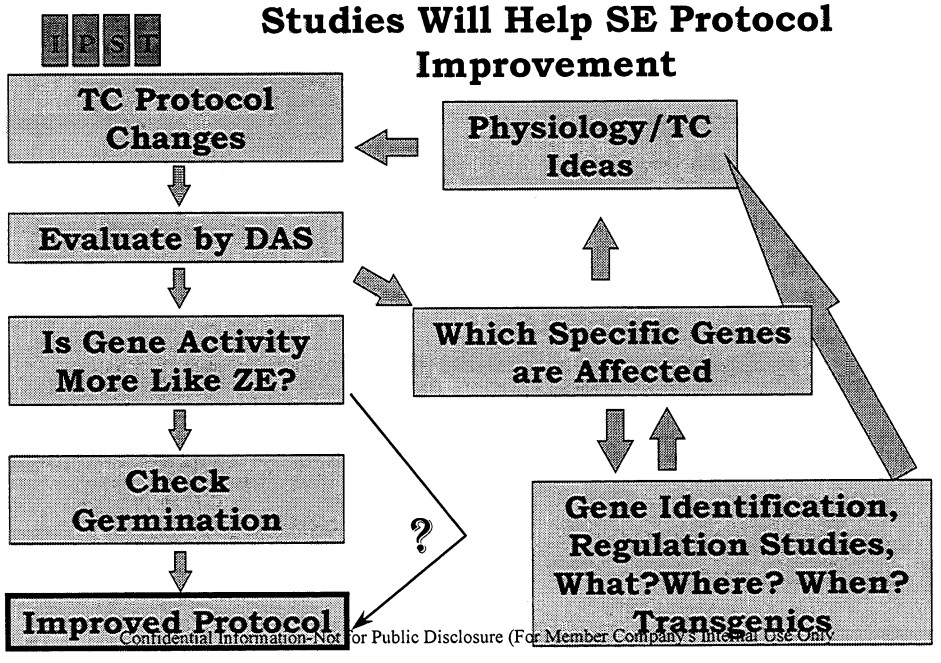
Topics of Today's Presentation

- Introduction: Rationale and Link between Somatic Embryogenesis and This Project
- Review Past Accomplishments
- Current Results
- New Goals

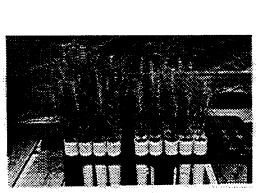
Introduction: Two Fundamental Reasons for Exploring Embryo Gene Activity

- **Immediate: Develop Hypotheses for Improving Somatic Embryogenesis Protocols**
- **Long Term: Illuminate Embryo Development**

Introduction: How Gene Expression Studies Will Help SE Protocol Improvement

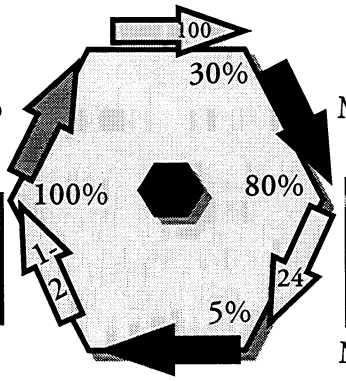


Introduction: Low Initiation Efficiency makes Early Stage a Target for Gene Expression Analysis

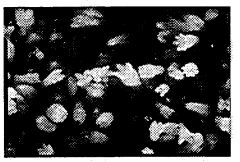


Initiation 15-30%

Conversion 80-100%



Multiplication 100%

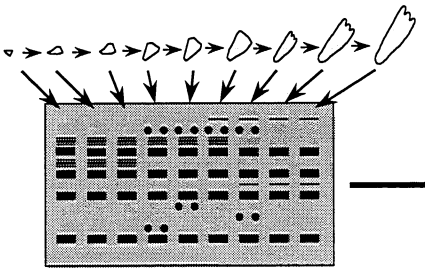


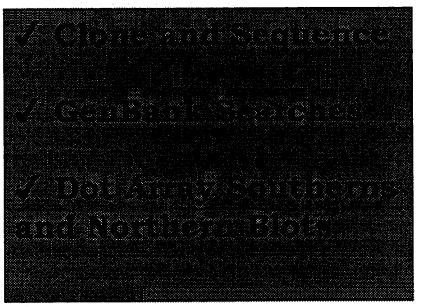
Germination 0-50%

Maturation 5-90%

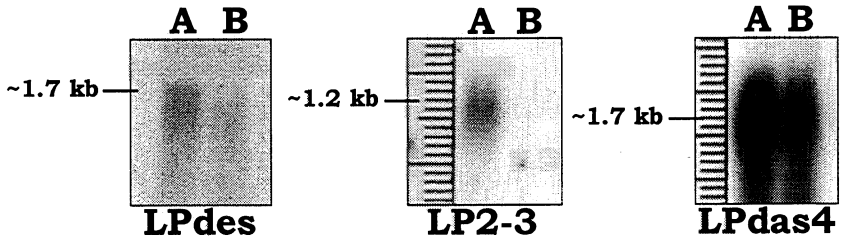
Review: Differential Display to Uncover Early Abundant cDNAs

✓ **Differential Display**





Review: Decided to Focus on 3 cDNAs for Detailed Study



A = Somatic Genotype 314 Liquid Suspension RNA
B = Somatic Genotype 314 Late Stage (6 - 9) RNA

New Results

- **Cloning of full-length cDNAs**
  - *DD bands are fragments; need whole cDNA for protein synthesis*
- **Repetition and Quantification of Northern Blots**
  - *Image analysis for quantifying difference in early versus late mRNA abundance*
- **Cloning and Sequencing LPdes Promoters**
  - *In preparation for promoter - GUS constructions*

New Results

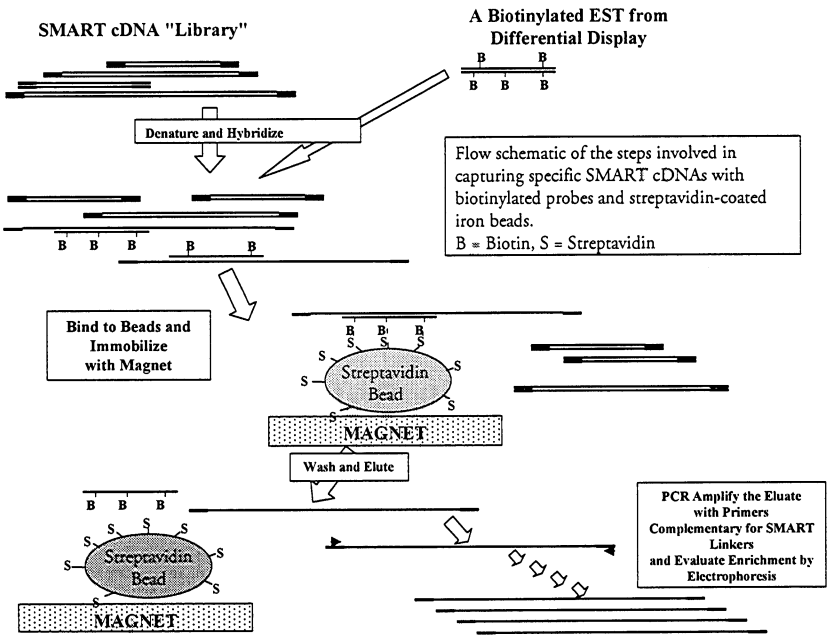
- **Cloning of full-length cDNAs**
  - *DD bands are fragments; need whole cDNA for protein synthesis*
- **Repetition and Quantification of Northern Blots**
  - *Image analysis for quantifying difference in early versus late mRNA abundance*
- **Cloning and Sequencing LPdes Promoters**
  - *In preparation for promoter - GUS constructions*

What do these genes do? What is their role in embryogenesis?

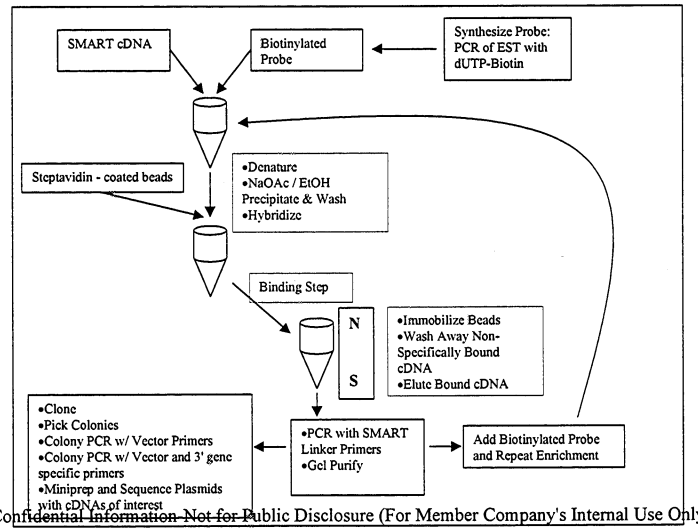
- **First,**
  Determine a Biochemical Function
- **Expressing a protein for the purpose of determining its function or generating Antibodies requires a single cDNA which contains the complete coding sequence.**

Development of New, Rapid Method for full-length cDNA Isolation

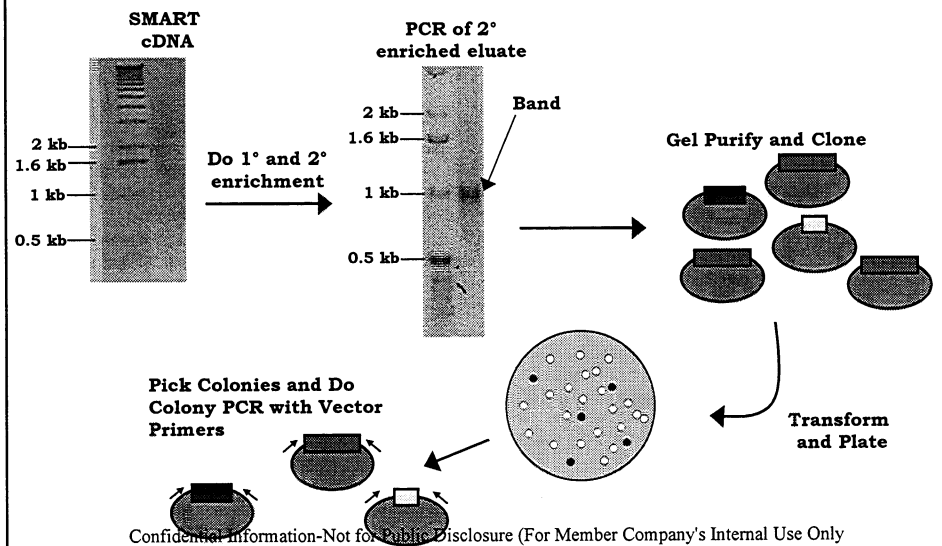
- **The construction, packaging and screening of cDNA libraries is a reliable method for clone isolation, but labor- and material-intensive.**
- **cDNA libraries (unpackaged) have been made, using the SMART cDNA method (Clontech), from zygotic head, suspensor and megagametophyte (John MacKay) and somatic liquid suspension culture (L. Destefano).**
- **I have developed a hybridization-bead extraction method for rapid selection of full-length clones from the library.**



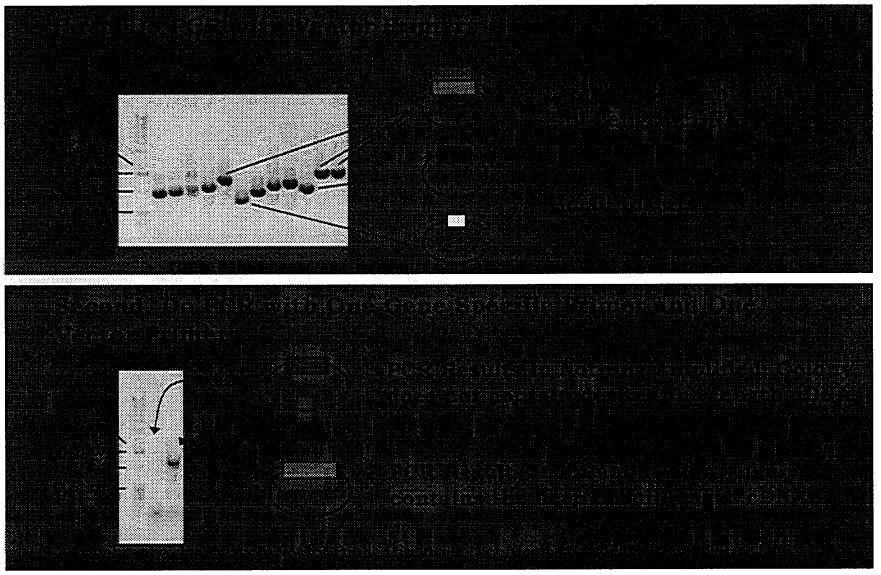
The full-length cloning method is an iterative process generally requiring at least two iterations



Example: LPZ195 full-length cloning



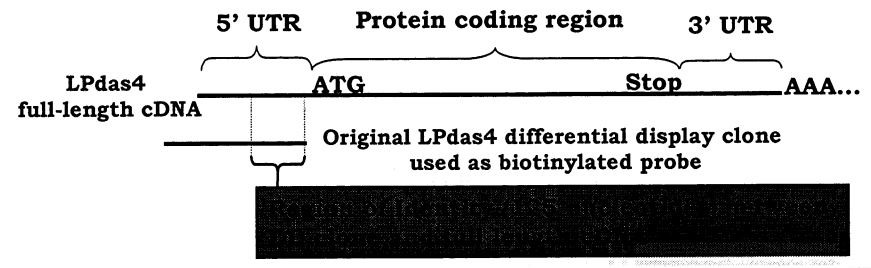
Colony PCR



Results with full-length cloning method

- **Captured full-length for LPdes, LPdas4, and LPZ195 (LP2-3 to follow shortly)**
- **Method has advantage of allowing for selection of the longest sequences**
- **Method appears amenable to enriching for multiple cDNAs in the same hybridization**
- **May be useful in identifying many of the "unknown" catalogued cDNAs from N. Xu**

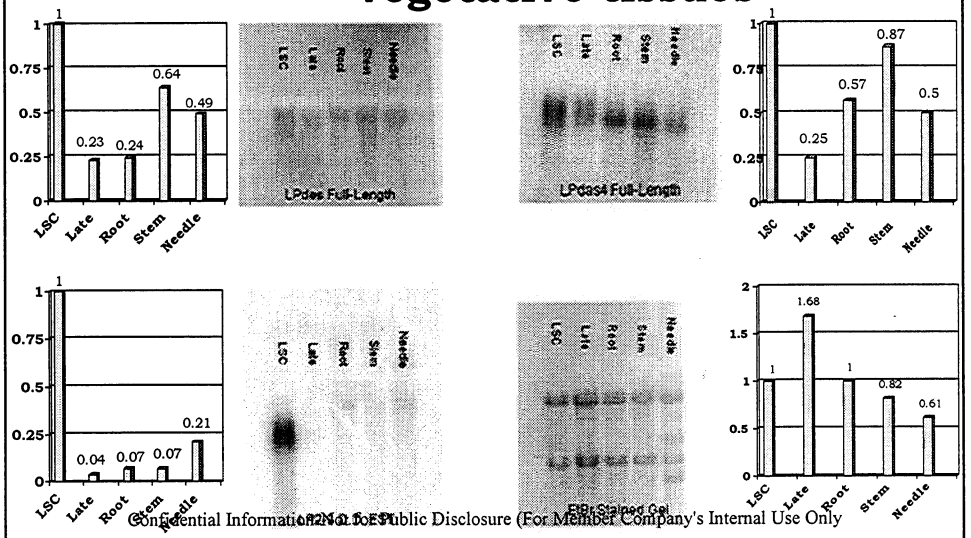
Example: LPdas4 identified after full-length capture



New Results

- **Cloning of full-length cDNAs**
  - *DD bands are fragments; need whole cDNA for protein synthesis*
- **Repetition and Quantification of Northern Blots**
  - *Image analysis for quantifying difference in early versus late mRNA abundance*
- **Cloning and Sequencing LPdes Promoters**
  - *In preparation for promoter - GUS constructions*

Northern Blots for LPdes, LPdas4, and LP2-3 versus embryo and vegetative tissues



New Results

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  - *DD bands are fragments; need whole cDNA for protein synthesis*
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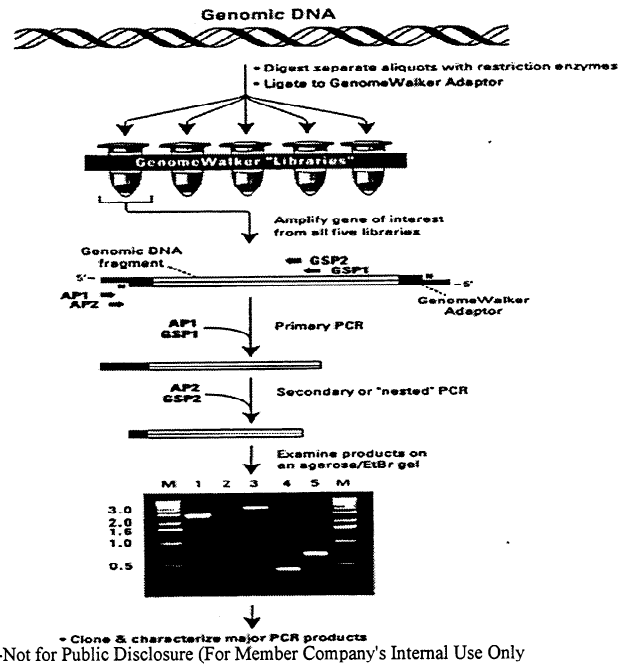
- **Cloning and Sequencing LPdes Promoters**
  - *In preparation for promoter - GUS constructions*

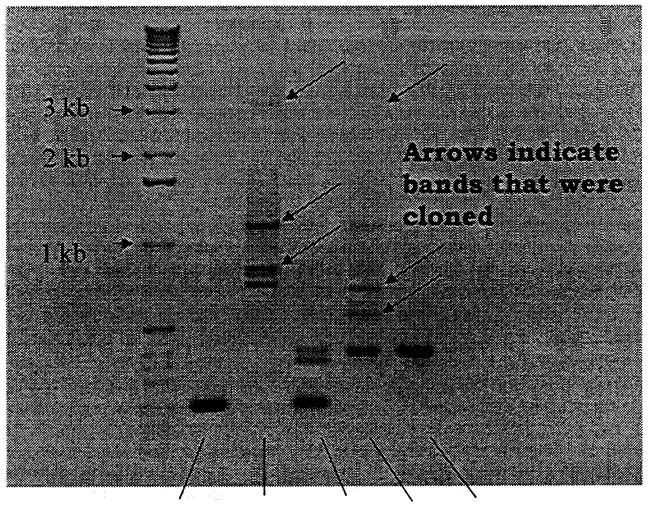
Promoter Isolation Gives Insight into Gene Regulatory Mechanisms

- **Promoters determine the synthesis of mRNA from genes**
- **Isolating promoters for stage-specific genes could allow us to express desirable proteins in a stage-specific manner**

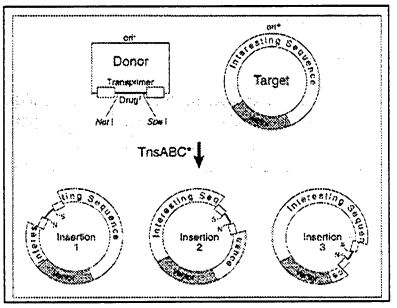
Rapid Isolation of Upstream Sequences (Promoters) by Genome Walker System (Clontech)

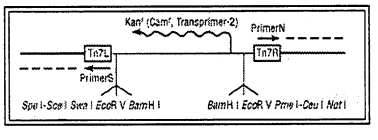


Isolation of LPdes Promoters by Genome Walker System



Genome Priming System (New England Biolabs) allows sequencing of Promoters greater than ~1000 bp





- **A single sequencing reaction can return ~500 bp**

Genome Priming System

- **Transposons (called transprimers) are randomly inserted in the cloned Genome Walker primer sequences by an enzyme combination, TnsABC.**
- **Transposons function as priming sites**
- **~3300 bp LPdes primer regions can be sequenced**

Promoter Sequencing Results

- So far, at least two different LPdes promoter sequences found; likely more
- Genome Priming System is well suited for purposes of sequencing promoters

Goals Towards Completion

- Assay for Desaturase Activity of LPdes by Expression and Fatty Acid Analysis
- Complete Expression Analysis in Embryo Tissue for LPdes, LPdas4, and LP2-3
- Create LPdes Promoter - GUS Fusions and Transform Appropriate Host

FIBER PROPERTY MODIFICATION

IPST DUES FUNDED RESEARCH CONSORTIUM
1998-1999

FUNDAMENTAL BIOLOGICAL MECHANISMS: IMPROVED STEM GROWTH RATES AND FIBER PROPERTIES

Status Report for
Project F011

Gary Peter
John Cairney
John MacKay
Gerald Pullman
Douglas Benton
Huabin Meng
Christina Perfetti

March 25-26, 1999

IPST-DRFC PROJECT SUMMARY 1998-99

- Project Staff
  - Faculty/Senior Staff: Gary Peter, John Cairney, John MacKay, Gerald Pullman
  - Staff: Postdoc (open)
- FY 98-99 Budget: 125,000
- Allocated as Matching Funds: 10%

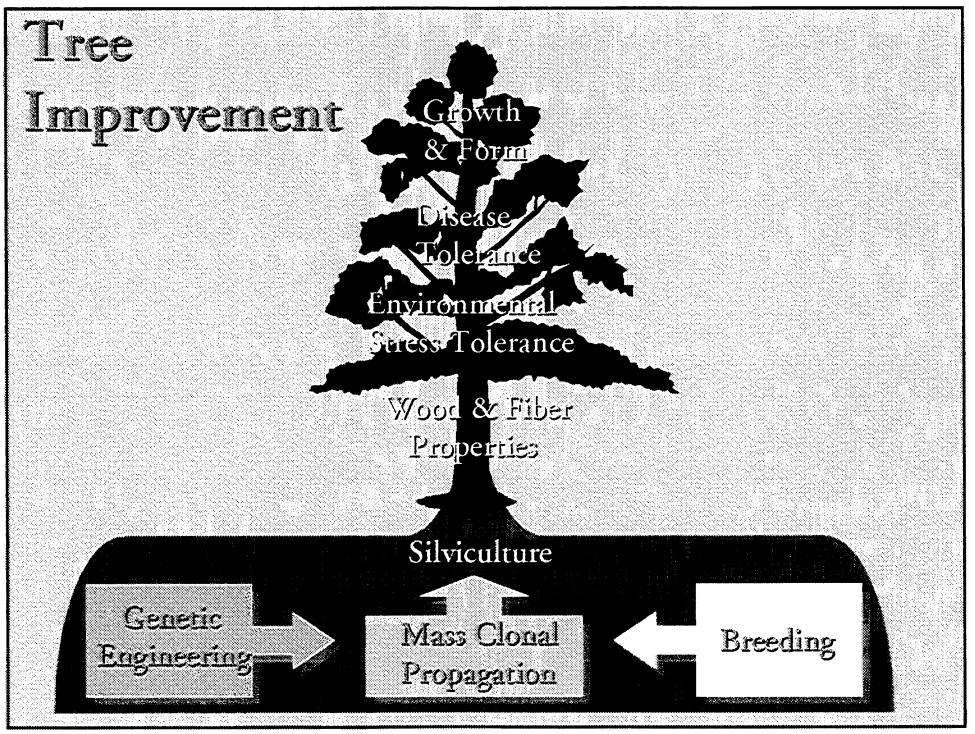
IPST-DRFC PROJECT SUMMARY 1998-99

- Time Allocation
  - Faculty/Senior Staff: 0.4
  - Support: 1.0
- Supporting Research
  - M.S. Student: Douglas Benton, Matt Roberts
  - Ph.D. Student: Michael Sullivan
  - External: Haubin Meng, open (postdoctoral fellows)

RESEARCH LINE/ROAD MAP

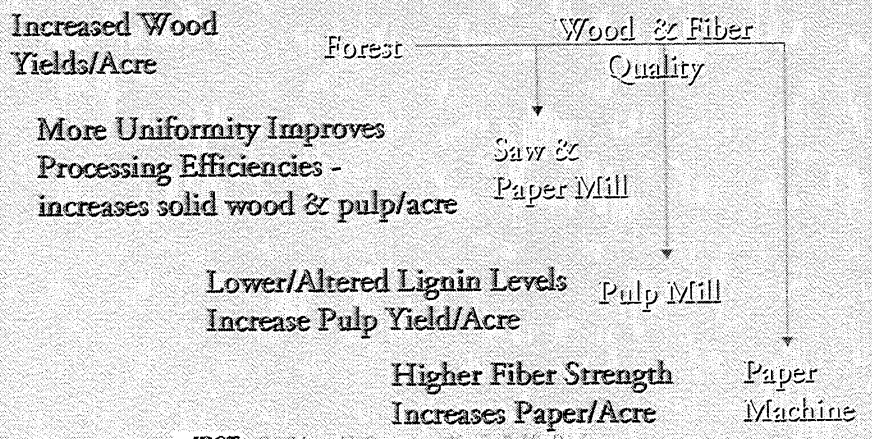
- *Area* - Improved Forest Productivity, Environmental Performance, Improved Capital Effectiveness, Energy Performance, Convertibility and End-use Performance
- *1º Research Line* - Develop fibers with properties similar to or better than Northern softwood and Eucalyptus that can be grown in most regions of North America
- *Road Map* - Develop fundamental understanding of secondary wall formation and the regulation of stem growth rates

IPST



Value of Tree Improvement

⇧ PRODUCT UNITS/ACRE = MORE $ FROM SMALLER LAND BASE



RESEARCH LINE/ROAD MAP

- *Area* - Improved Forest Productivity, Environmental Performance, Improved Capital Effectiveness, Energy Performance, Convertibility and End-use Performance
- *1º Research Line* (#2)- Develop fibers with properties similar to or better than Northern softwood and Eucalyptus that can be grown in most regions of North America
- *Road Map* - Develop fundamental understanding of secondary wall formation and the regulation of stem growth rates

PROJECT OBJECTIVES

- This project has three broad objectives:
  - 1) increase the growth rate of the stem
  - 2) improve fiber properties for value added paper products
  - 3) improve the processing characteristics of wood to decrease environmental impacts while increasing fiber yield and quality

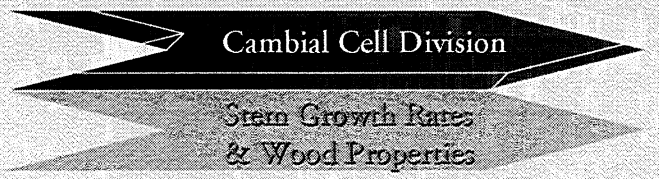
GOALS FOR FY 98-99:

- Complete standardization of tissue culture and transformation methods for *P. deltoides* C175.
- Isolate cyclin cDNAs that are expressed in the cambial meristems of Poplar and/or loblolly pine.
  - A) Structurally characterize cDNAs
  - B) Begin experiments to characterize their patterns of expression within the cambial meristem
  - C) Begin to determine spatial patterns of gene expression within the stem for specific cyclins.

Approaches to Increase Stem Growth Rate

Molecular Genetic

- Cell Cycle Regulation - Isolate & Characterize cambial cyclin cDNAs
- Overexpress cyclin cDNAs to stimulate cell division rates



Hypothesis: More cambial cell divisions = more fibers per volume of wood with thinner cell walls

IPST

GOALS FOR FY 98-99:

- Isolate celA cDNA(s) from *Pinus taeda.*
  - A) Structurally characterize cDNAs
  - B) Begin experiments to characterize the pattern(s) of expression within the differentiating xylem
- Isolate full length rac GTP-binding protein cDNAs from *Z. elegans.*
  - Begin structural characterization of cDNAs (This work will be done depending upon progress made on the above goals)

IPST

Approaches to Lower MFA in Juvenile Wood

Genetic

- Accurate, Rapid, Economical Method to Measure MFA in Southern Pine
- Identify Mutants in Model Plants

High MFA → Microfibril Angle → Low MFA

Wood & Fiber Properties

Biochemical

- Mechanism of Cellulose Synthase Interaction with Microtubules
- Regulators of Microtubule Organization

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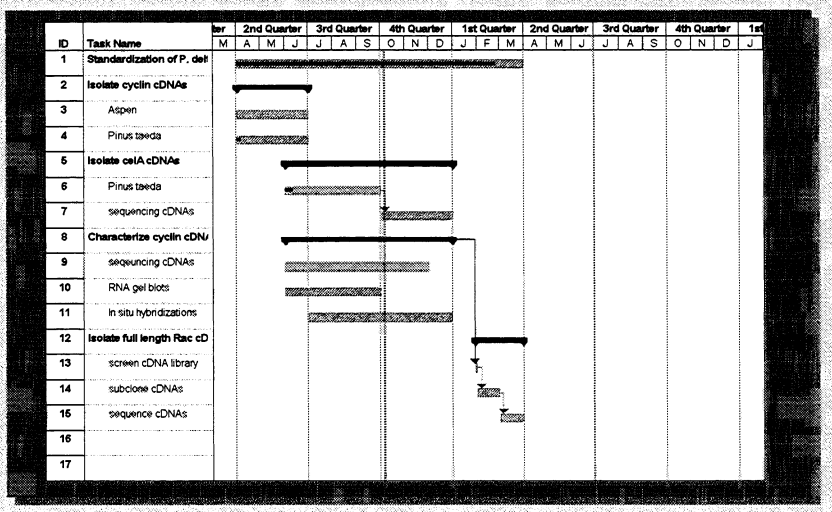
Gantt Chart for FY 98-99



IPST

PROJECT OBJECTIVES

- This project has three broad objectives:
  - 1) increase the growth rate of the stem
  - 2) improve fiber properties for value added paper products
  - 3) improve the processing characteristics of wood to decrease environmental impacts while increasing fiber yield and quality

Deliverables For FY 99-00: Regulation of Stem Growth Rates

- Isolate cyclin cDNAs that are expressed in the cambial meristems of loblolly pine.
  - A) Structurally characterize cDNAs
  - B) Begin experiments to characterize their patterns of expression within the cambial meristem
  - C) Begin to determine spatial patterns of gene expression within the stem for specific cyclins.

(Pending funding from AF&PA/DOE/USFS -Agenda 2020)

Deliverables for FY 99-00: Genetic & Environmental Control of Wood Properties

- Measure the MFA and fiber lengths from pith to bark and base to tip of southern pine trees from intensively managed plantations
- Develop rapid method for measuring *stiffness* in wood core samples (*pending funding from TIP3/State of Georgia*)
- Initiate genetic mapping of wood quality traits - MFA, fiber length and coarseness in loblolly pine (*pending funding from NSF Genome Panel*)

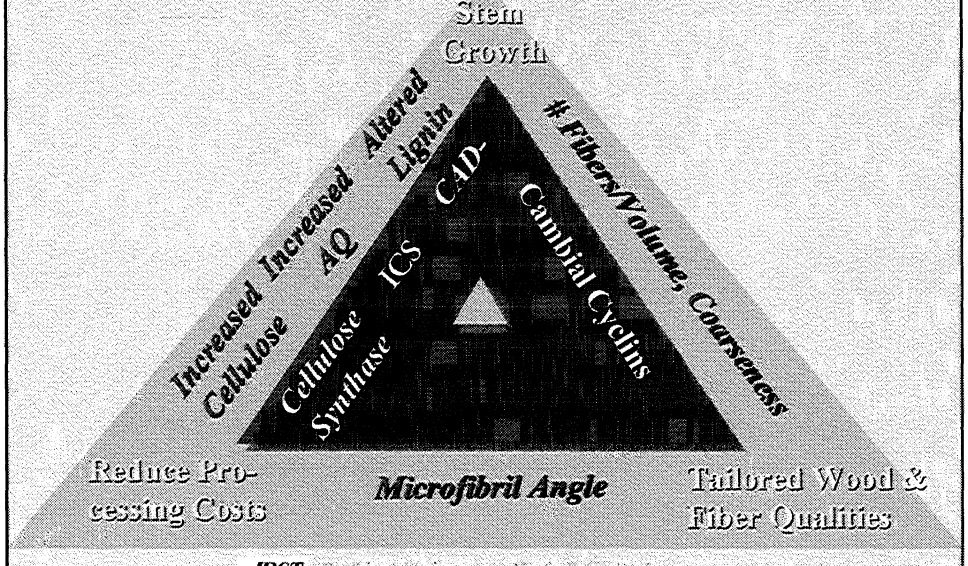
Deliverables For FY 99-00:

- Isolate celA cDNA(s) from *Pinus taeda.*
  - A) Structurally characterize cDNAs
  - B) Begin experiments to characterize the pattern(s) of expression within the differentiating xylem
  - C) Create transformed cell lines that overexpress a tagged celA gene for characterizing additional subunits and testing for elevated levels of cellulose

Deliverables for FY 99-00: Genetic Engineering for Improvement of Loblolly Pine

- Isolate gene regulatory sequences that promote constitutive expression
- Test the function of gene regulatory sequences
- Use these sequences to build efficient positive and negative selection systems
- Create transformed embryo lines with visible markers linked to ABA and osmotic regulatory sequences
  (*Pending funding from TIP3/State of Georgia*)

Goals of F011 & Related Projects



Student Research

- Michael Sullivan - PhD candidate
  - Developing antibodies for glucuronoxylan backbone and side chains
  - Use to characterize xylan location and to develop assay for β 1,4-xylan synthase
- Douglas Benton MS (2nd year)-
  - Complete validation of DIC method with confocal method
- Matthew Roberts MS (1st year) -
  - Complete MFA and fiber length measurements and analysis from managed sites

IPST

External Funding FY 98-99

• External Funds	281,845
– *AF&PA-DOE 2020 (AQ)*	175,845
– *TIP³ (MFA)*	27,000
– *TIP³ (Pine Transformation)*	55,000
– *USDA (CAD⁻ Lignin)*	24,000

EXTERNALLY FUNDED RESEARCH in 1998-1999 SUPPORTING F011

Gary Peter
John Cairney
John MacKay
Gerald Pullman
Don Dimmel
Elizabeth Althan
Douglas Benton
Luis Destefano
Huabin Meng

March 25-26, 1999

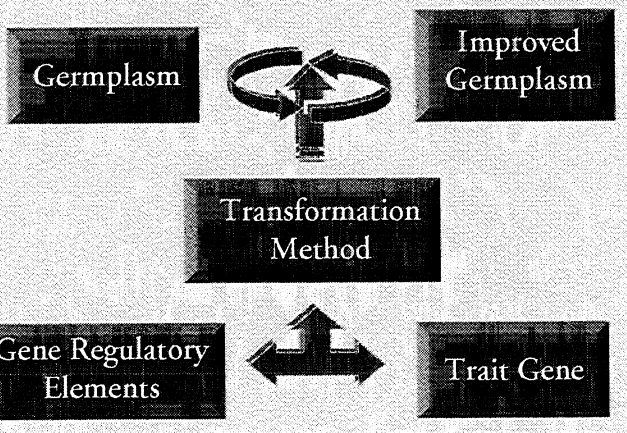
TIP^3

Genetic Transformation of *Pinus taeda*

- Efficient transformation methods for loblolly pine

Gary Peter, Luis Destefano, Alan Wenck,
Teresa Vales, John Cairney, Jerry Pullman

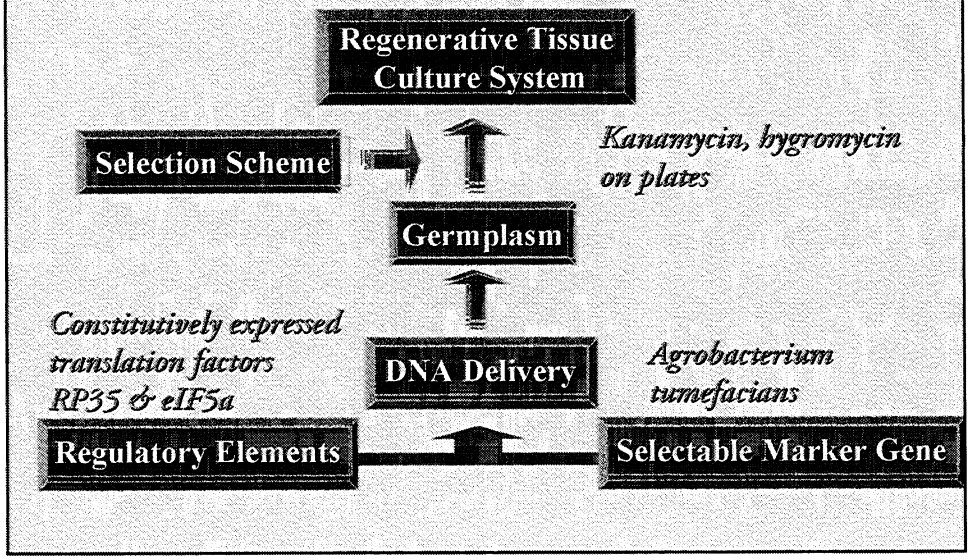
Tree Improvement by Genetic Engineering



Goals and Objectives

- Develop efficient transformation methods for *Pinus taeda*
  - optimize DNA transfer
  - optimize selection
  - insure constitutive expression
  - optimize regeneration of transformants

Core Enabling Technologies for Genetic Engineering



DNA Transfer with *Agrobacterium tumefacians*

- *Agrobacterium tumefacians* can transfer DNA into early stage embryos of loblolly pine
- Presence of extra *vir B* and *vir G* genes improves transfer efficiencies
- Transfer requires acetosyringone induction of *vir* genes

Strain	Spruce	Loblolly Pine
EHA 105 (pBISN1)	152 ± 88*	11 ± 5
EHA 105 (pBISN1, pToK47)	1224 ± 199	121 ± 34
GV 3101 (pBISN1)	470 ± 108	21 ± 21
GV 3101 (pBISN1, pToK47)	410 ±122	6 ±1
LBA 4404 (pBISN1, pToK47)	459 ± 69	29 ± 15

Acetosyringone (uM)	Spruce[1]	Loblolly Pine[2]
0	58 ± 583	0
25	574 ± 90	41 ± 4
50	512 ± 38	97 ± 60
100	276 ± 152	95 ± 3

1. LBA 4404 (pBISN1, pToK47) was used
2. GV 3101 (pBISN1, pToK47) was used
3. The # of GUS positives were scored after 2-3 days

Selection of Transformed Embryos

LIQUID

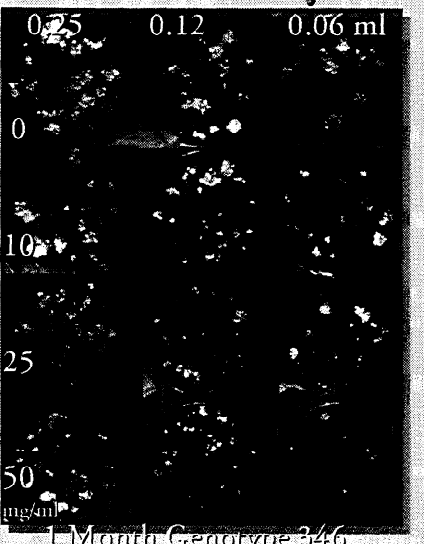
- Efficient selection at minimal drug concentrations due to immersion in drug
- Doesn't allow for isolation of individual transformants

SOLID

- Selection may not be efficient at low drug levels
- Allows for selection of individual transformants before maturation
  - Involves plating at reduced densities

Kanamycin Concentrations for Selection of Transformed Embryos

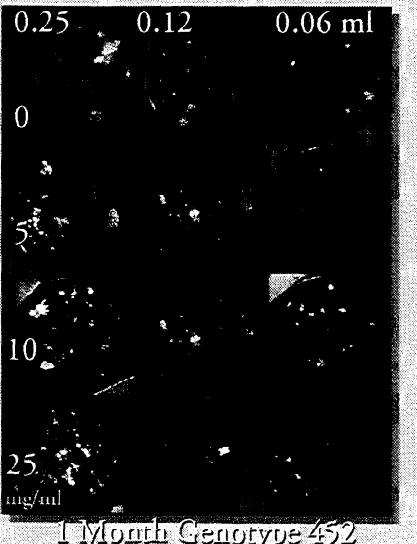
- Plating experiments varied cell density and kanamycin concentration
- 50 mg/ml is not enough drug at higher plating densities
- 50 mg/ml may be enough at low densities
- Need to test higher kanamycin levels



1 Month Genotype 346

Hygromycin Concentrations for Selection of Transformed Embryos

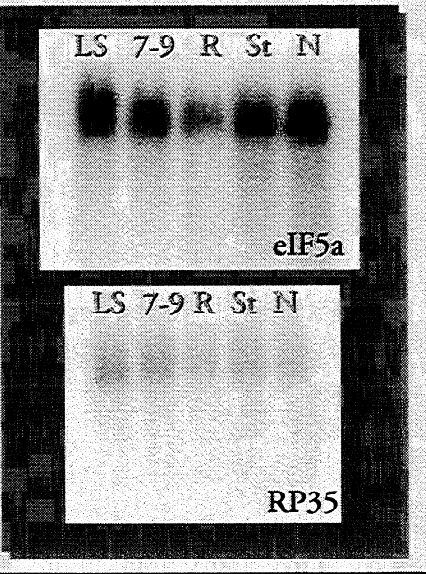
- Plating experiments varied cell density and hygromycin concentration
- 25 mg/ml is not enough drug at higher plating densities
- 25 mg/ml may be enough at low densities
- Need to test higher hygromycin levels



1 Month Genotype 452

Constitutively Expressed Loblolly Genes: Potential Promoters for Efficient Selections

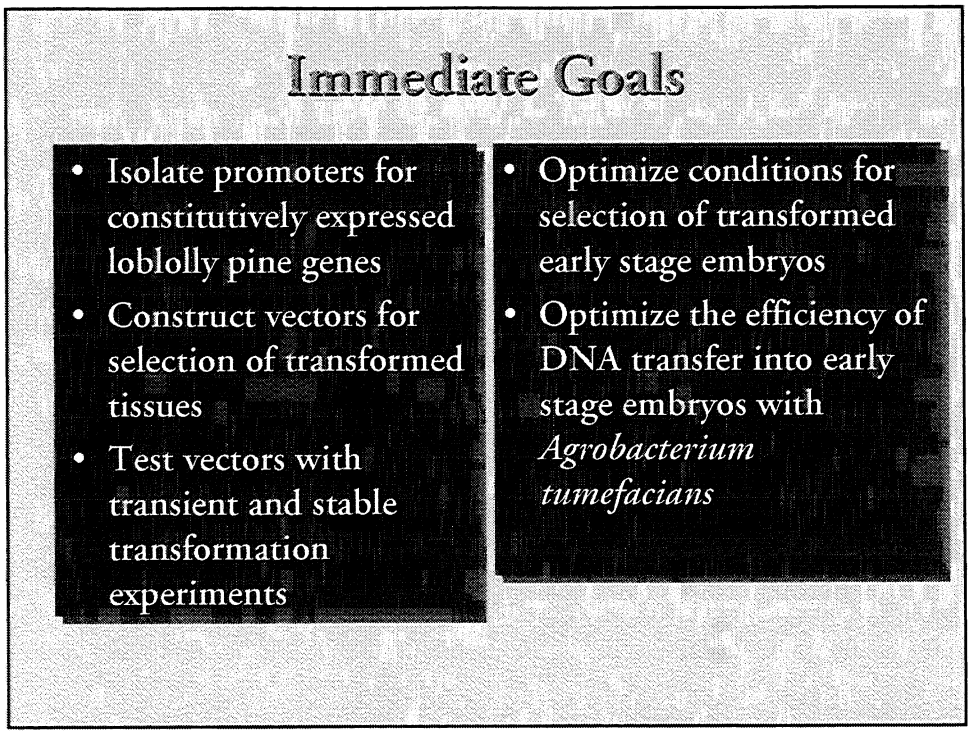
- Two partial cDNAs show constitutive expression
- These cDNAs code for proteins that are involved in translation



Isolation of Full Length cDNAs to Aid in Promoter Isolation

- cDNA libraries were constructed from total RNA isolated from
  - early stage embryos cultured in liquid suspension
  - somatic embryos stages 7-9
  - stems

Tissue	Total # of Phage	% Recombinants
Liquid Suspension (1-2)	3.0×10^6	95
Somatic Embryos (7-9)	8.0×10^6	97
Stems	1.3×10^6	91


Immediate Goals
• Isolate promoters for constitutively expressed loblolly pine genes
• Construct vectors for selection of transformed tissues
• Test vectors with transient and stable transformation experiments
• Optimize conditions for selection of transformed early stage embryos
• Optimize the efficiency of DNA transfer into early stage embryos with *Agrobacterium tumefacians*

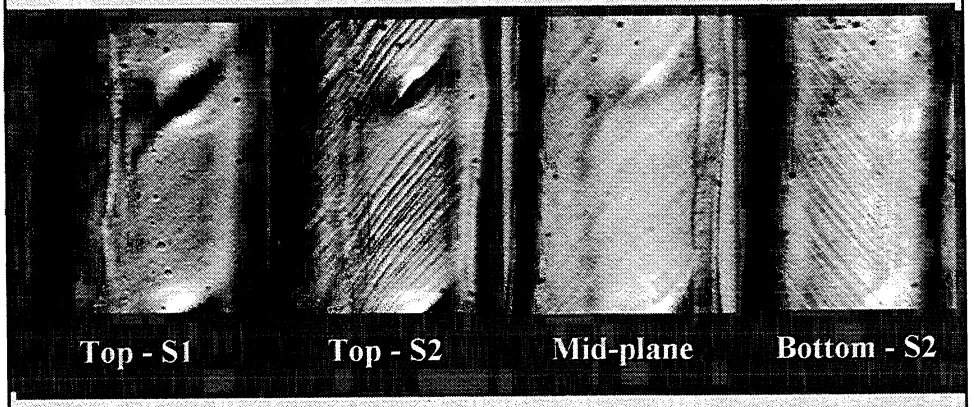
TIP³

New Method for MFA Measurement

- High resolution optical microscopy with differential interference optics can be used to visualize the MFA of the S2 layer in thick walled Southern Pine tracheids.

Gary Peter, Douglas Benton, Matthew Roberts
Keith Bennett - Weyerhaeuser Co

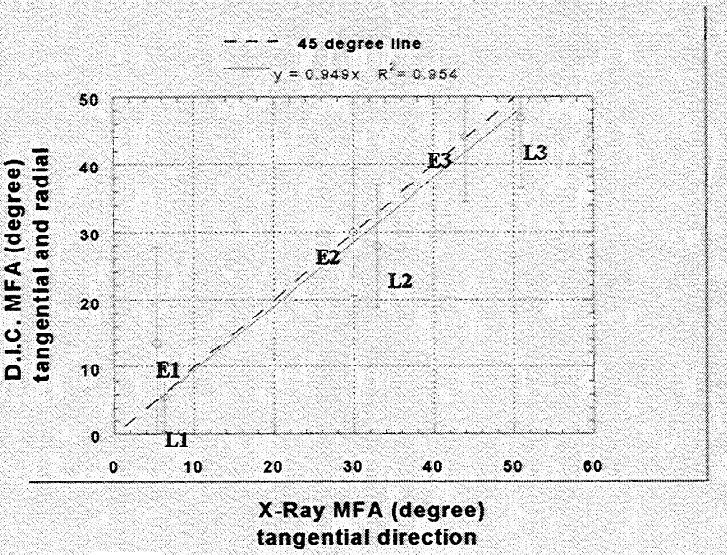
DIC Image of Loblolly Pine Tracheid



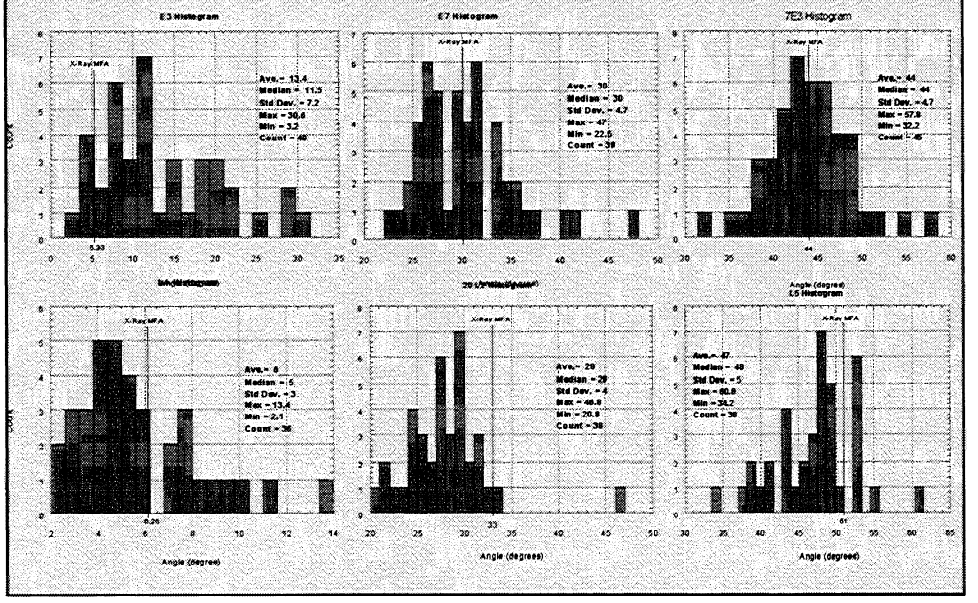
Goals and Objectives

- Validation of Differential Interference Contrast Microscopy as an Accurate Method of M.F.A. Determination
- Determination of the Number of Fibers that Must be Measured to Produce an Accurate Mean M.F.A. from Pith to Bark within a Single Tree
- Determine the M.F.A. from Plantation Grown Pines in Maximized Growth Environments

D.I.C. Verification



M.F.A. Distributions



of Measurements/Sample for Valid Mean MFA

- Four microfibrils/fiber are measured and averaged
- A sample size of 10 fibers (40 measurements) gives a good mean value

Sample A Results

Sample size	Average	Std. Dev.	Range
5	42.2	1.8	40-45
10	41.8	2.4	38-45
15	41.3	3.4	34-47
20	40.5	3.2	34-47

Previously Recorded Results of Sample A

Sample Size	Average	Std. Dev.	Range
40	44	4.7	32-58

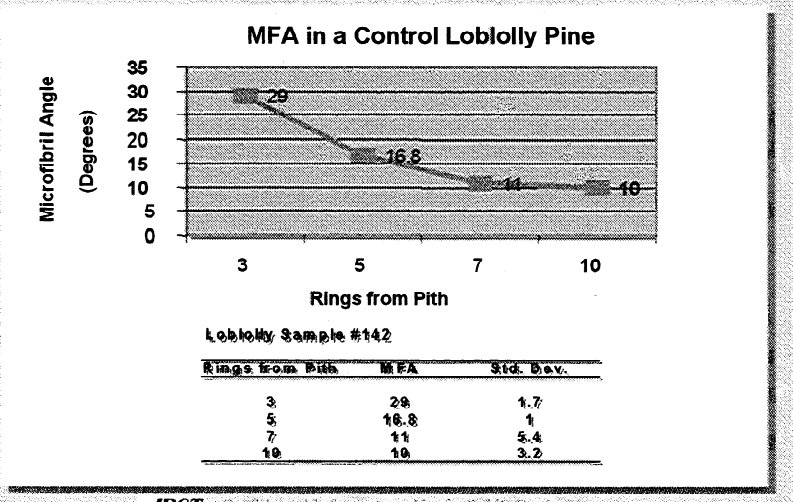
Sample B Results

Sample Size	Average	Std. Dev.	Range
5	31.35	3.94	29-37
10	29.12	4.19	24-37
15	29.81	4.55	24-40
20	29.64	4.20	24-40

Previously Recorded Results of Sample B

Sample Size	Average	Std. Dev.	Range
40	30	4.7	22.5-39

Latewood MFA in Control Loblolly Pine



Loblolly Sample #142

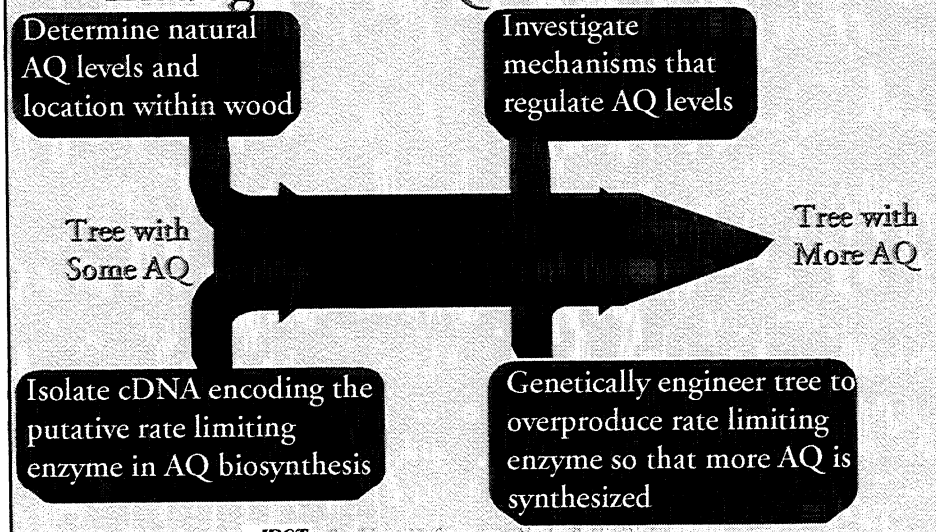
Rings from Pith	MFA	Std. Dev.
3	29	1.7
5	16.8	1
7	11	5.4
10	10	3.2

Agenda 2020

Endogenous Anthraquinone Pulping Catalysts

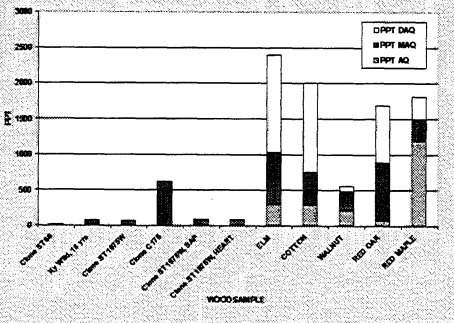
Gary Peter, Don Dimmel, Jerry Pullman
Huabin Meng, Perla Seklar, Karen Crews

Strategy to Increase the Level of Endogenous AQs in Cottonwood



Endogenous AQ Levels in Trees

- AQ is present in hardwood trees; however, at much lower levels than previously reported PPT vs. %'s
- C175 has ~600 PPT monomethyl AQ
- methyl anthone - new endogenous pulping catalyst



Cloning of Isochorismate Synthase: The Putative Rate Limiting Enzyme for AQ Biosynthesis

- A putative full-length isochorismate synthase cDNA was isolated from *Arabidopsis thaliana*
  - high sequence similarity to other bacterial and eucaryotic ICSs
  - cDNA encodes for ~ 55 kDa protein

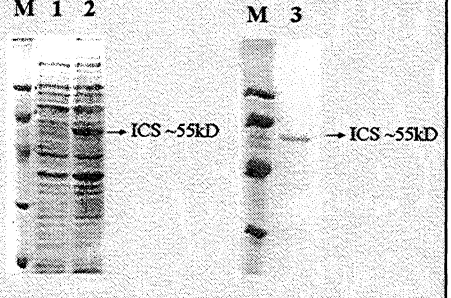
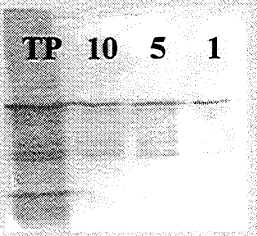


Fig 1. Expression and purification of arabidopsis ICS in E. coli. Lane1 shows protein profile of E.coli control with no arabidopsis ICS gene. Lane2 show the expression of arabidopsis ICS in E.coli. Lane3 shows the purified ICS protein. Lane M are marker protein.

Regulation of the ICS Gene & Endogenous AQ Levels

- To investigate what cell types produce the ICS enzyme we expressed a His-tagged fusion in E. coli and have produced high titer polyclonal antibodies against the purified protein

Ab Dilution	# 3524 PI	#3524 I	#3525 PI	#3524 I
1:10,000	0.104	>2.0	0.092	>2.0
1:100,000	0.084	1.386	0.089	1.821



Genetic Engineering the Overexpression of ICS in Cottonwood

Regulation of ICS

- ICS induced by elicitors
- ICS mRNA present in roots of Arabidopsis
- ICS induced by wounding in leaves
- ICS expression in cells with enzymes leading to AQ; heartwood

Why Overexpress ICS?

- Elevate endogenous AQ levels
- Reduce flux into lignin monomer pathway
- Increase systemically the level of salicylic acid; an inducer of plant defense responses

Anthraquinone Biosynthesis

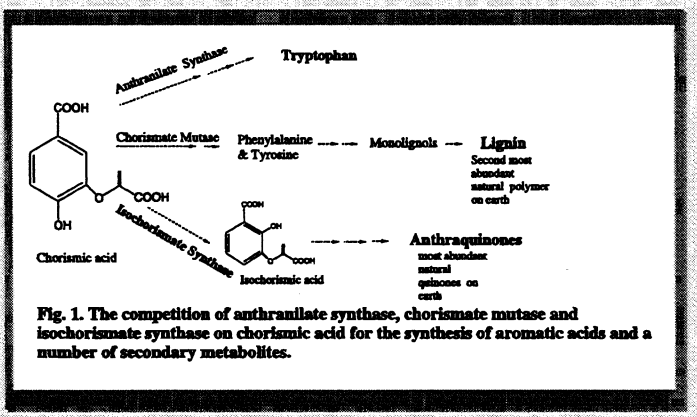


Fig. 1. The competition of anthranilate synthase, chorismate mutase and isochorismate synthase on chorismic acid for the synthesis of aromatic acids and a number of secondary metabolites.

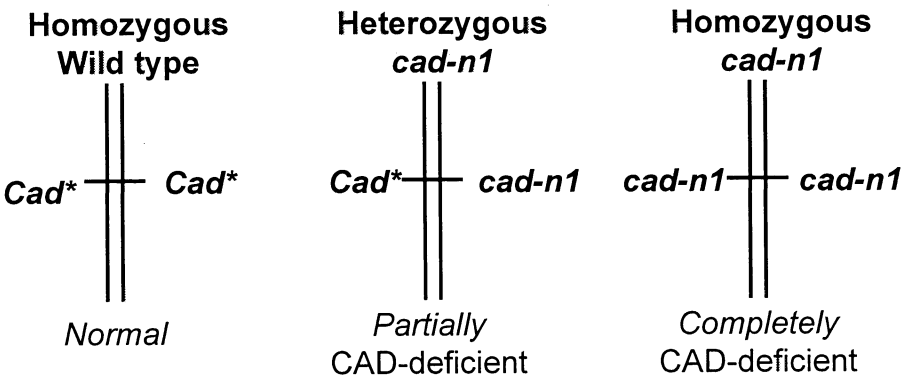
Genetic Engineering the Overexpression of ICS in Cottonwood

- The nearly full-length ICS cDNA has been introduced into a plant transformation vector
- This vector has been mobilized into *Agrobacterium tumefacians* for transformation of *Populus deltoidies* C175

CAD-Deficient Trees

- Funding: USDA-NRI, in conjunction with NC State University: IPST is a subcontractor ($50,000 October 97-99)
- Personnel: Elizabeth Althen (Sr Tech), Christy Parks (Summer Intern), Don Dimmel, John MacKay
- Goal: The ultimate goal is to develop trees that are easily delignified without using gene transfer technology.
- Specific objectives: Characterize the reactivity and lignin removal of woods from CAD-deficient trees. Understand the relationship between reactivity and altered lignin structure.

Allelic Variation at the *cad* Locus



*** Represents any wild type allele of *Cad***

Completely vs. Partially CAD-Deficient Trees

Completely **CAD-Deficient**

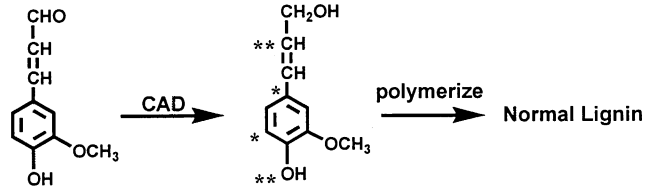
- Dramatically altered lignin structure.
- Lignin removal is greatly simplified.
- Model to study reactivity and removal of altered lignin in extreme case.
- Limited growth potential

Partially **CAD-Deficient**

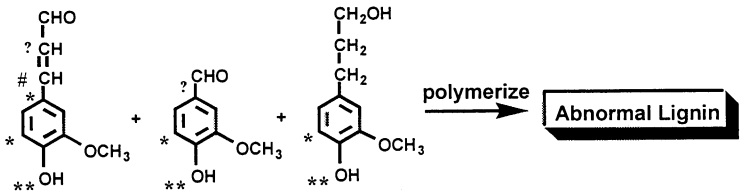
- Potential commercial value for production.
- Partially CAD-deficient trees have rapid growth: 14% more volume than the "normal trees" after 4 years of growth.
- Expected to pulp to more efficiently lower Kappa.

Modified Pool of Lignin Precursors

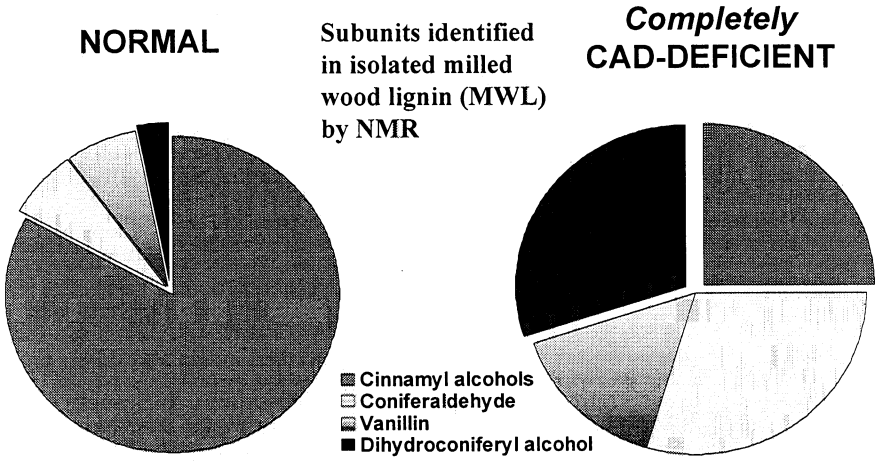
- Normal pine lignin biosynthesis:



- ***Completely*** **CAD-deficient pine lignin precursors:**



Distribution of Lignin Subunits



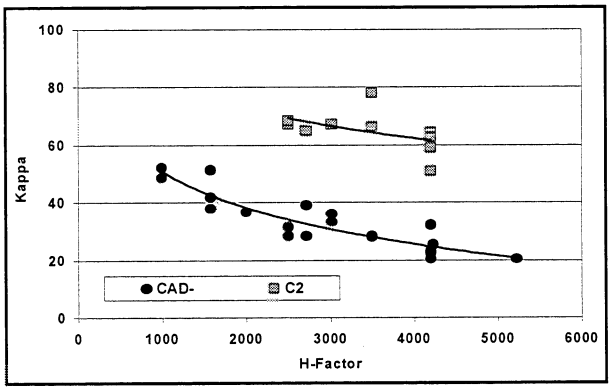
Altered Lignin Structure

- Fewer β - O_4 linkages because of monomers having inactive/no C_β
- More C_5 - C_5 and C_5 - O_4 linkages
- Expect CAD-deficient wood would be hard to delignify, unless the lignin:
  - Has a lower molecular weight
  - Contains more phenolic hydroxyl groups
  - Is less branched
  - Contains more ionic groups (-CHO -> $-COO^-$)

Experimental Goals

- **Determine the difference in pulping ease for CAD- and normal wood**
  - Pulping systems: Soda, Kraft, Soda/AQ
  - Effect of cook severity (H-factor, %NaOH)
  - Effect of additives (NaSH and AQ)
- **To determine the difference in bleaching ease**
- **To determine the reasons for any observed differences in pulping and bleaching by**
  - Correlating with lignin structure differences
  - Correlating with lignin mol. wt. differences
  - Kappa number (measure of residual lignin) and yield
  - Yield, purity, and mol. weight of dissolved lignins

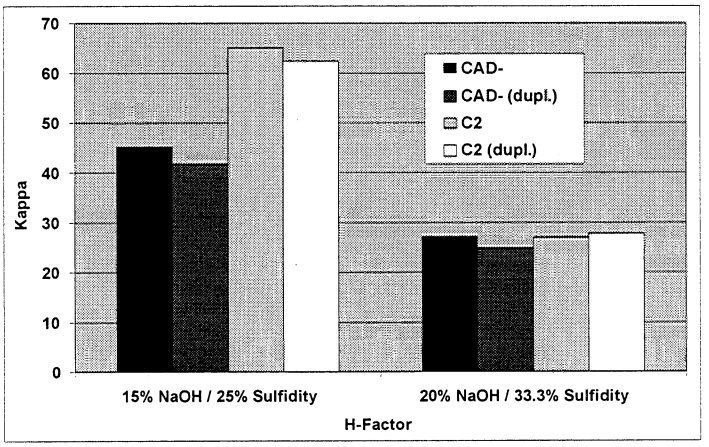
Soda Pulping of CAD-Deficient and Normal Pine Wood



- **Soda Pulping**
  - **18% NaOH**
- **Kappa #:**
  - **Residual lignin**
- **H-Factor**
  - **Cook severity**
  - **Time at 170 C**

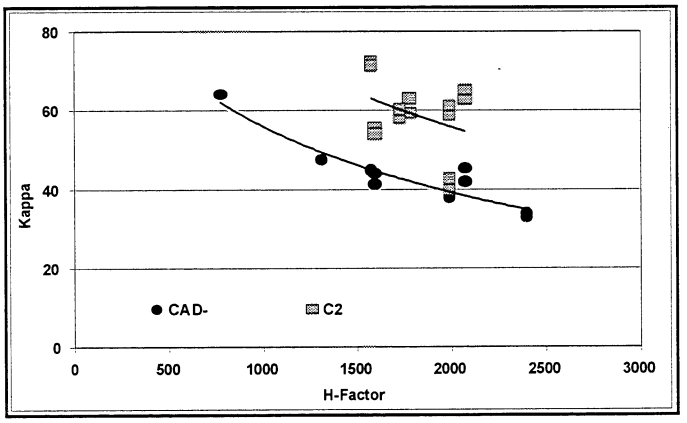
C2: Control **CAD-: CAD-Deficient**

Kraft pulping:
Effects of Pulp Chemical Concentration

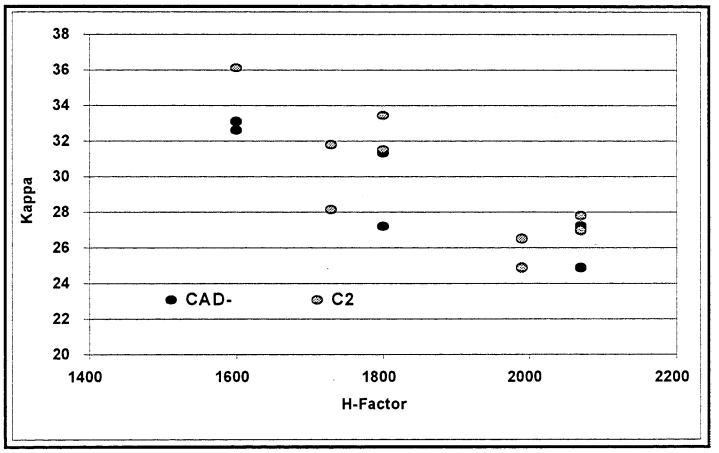


Kraft Pulping:
with 15% NaOH, 25% Sulfidity

"Mild" kraft conditions



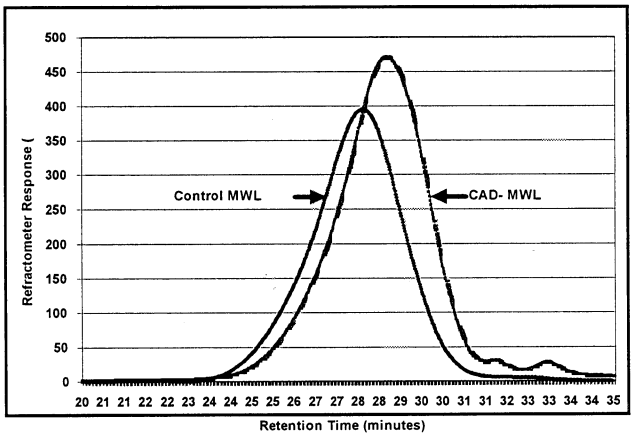
Kraft Pulping: with 20% NaOH, 33% Sulfidity



Are the Kappa Numbers Real?

- **The different types of linkages and increasee aldehyde content do not appear to affect the kappa determination.**
  - CAD- and normal MWLs consume the same amount of $KMnO_4$/gram (Jiebing Li, KTH)
- **Lingin isolation from black liquor: more lignin is recovered for CAD- than from normal pulping liquors.**

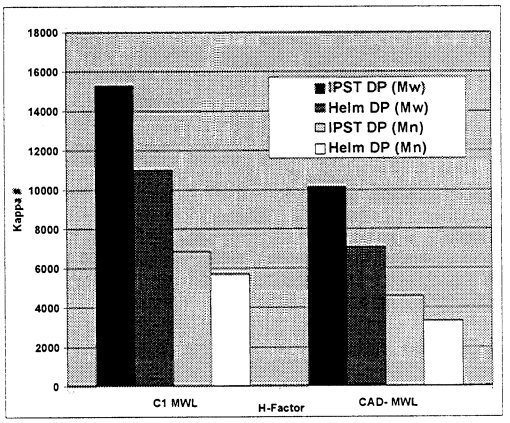
Molecular Weight of Milled Wood Lignin,
Determinations by IPST



Mol. Weight: Higher ⟶ Lower

Molecular Weight Determinations with Milled Wood Lignin

- **Determinations carried out by**
  - IPST
  - VA Tech (Helm)
- **CAD-deficient lignin is of lower molecular weight**
- **Mw: mean weight**
- **Mn: mean number**



MWL: Lignin isolated from wood C1: Control tree CAD-: CAD-deficient

Pulping Studies Conclusions

- **CAD- wood is much more easily delignified under mild conditions**
  - Soda and "mild" kraft
  - Reduced requirement for added NaSH
- **Reactivity of CAD- wood is consistent with new findings in lignin structure (Other research groups)**
- **Is the reactivity of CAD- wood due to a lower molecular weight lignin?**

Future Studies/ Directions

- **Comparative bleachability study of CAD- and normal, with 30-kappa pulps**
- **Quantitative analysis of the effects of NaOH, NaSH, and AQ on pulping CAD- wood**
- **Investigate relationship between lignin structure and CAD- pulping and bleaching reactivity**
- **Initiate pulping studies of *partially* CAD- wood**
- **Future studies should continue to exploit the natural variability that exist in lignin synthesis**
- **Grant proposals submitted to Agenda 2020 and USDA-NRI**